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THE ANAEROBIC DISSIMILATION OF D-GLUCOSE-1-C^{14}, D-ARABINOSE-1-C^{14}, AND L-ARABINOSE-1-C^{14} BY *AEROBACTER AEROGENES*[1]

BY A. C. NEISH AND F. J. SIMPSON

Abstract

D-Glucose-1-C^{14}, D-arabinose-1-C^{14}, and L-arabinose-1-C^{14} were dissimilated anaerobically by *Aerobacter aerogenes*. The major products (2,3-butanediol, ethanol, acetic acid, lactic acid, formic acid, and carbon dioxide) were isolated and the location of C^{14} determined. The products from glucose were all labeled, mainly in the methyl groups, in agreement with the hypothesis that they were derived from methyl-labeled pyruvate formed by the reactions of the classical Embden–Meyerhof scheme for glycolysis. The products from both pentoses appeared to have been formed from pyruvate labeled in both the methyl and carboxyl groups with twice as much C^{14} in the methyl group as in the carboxyl group. This result may be explained quantitatively by a hypothesis assuming complete conversion of pentose to triose via a heptulose.

Introduction

Certain bacteria are well known for their ability to utilize a variety of sugars as energy sources. *Aerobacter aerogenes*, for example, has been shown to ferment at least six of the eight aldopentoses (22). An explanation of the mechanisms involved should be of interest and aid in the understanding of basic problems in metabolism of living cells. This paper is concerned with the fermentation of 1-C^{14}-labeled D- and L-arabinose. These two sugars were chosen because they differ in the configuration of all three asymmetric carbon atoms and thus might be expected to be metabolized by widely different routes.

Experimental

Preparation of Labeled Compounds

The D-arabinose-1-C^{14} was prepared from D-erythrose by the cyanhydrin reaction. The D-erythrose solution was obtained by the Ruff degradation of

[1] *Manuscript received December 4, 1953.*

Contribution from the Prairie Regional Laboratory, National Research Council, Saskatoon, Saskatchewan. Issued as N.R.C. No. 3233. A report of this work was given at the Annual Meeting of the Society of American Bacteriologists, San Francisco, August 9–14, 1953.

calcium-D-arabonate, and purified by deionization with Amberlite IR-120 and IR-4B resins (4). The aldose content was determined by alkaline iodine (9) and found to be 35% of theoretical. The erythrose was treated with an equimolecular amount of $NaC^{14}N$ in a sodium carbonate solution as described by Isbell *et al.* (15) for the preparation of barium-D-gluconate. Calcium-D-arabonate was isolated by using carrier and obtained in about 30% yield. Subsequent work (19) has shown that yields of 59% are possible when a pure erythrose solution made by another method is used. The calcium-D-arabonate-1-C^{14} was converted to the lactone by passing an aqueous solution through an IR-120-H column followed by evaporation on a steam bath. The sirupy lactone was reduced with sodium amalgam in an oxalate buffered medium (15). The yield of aldose was only 40% but this could probably be improved by a study of the conditions for lactonization and reduction.

The L-arabinose-1-C^{14} was prepared by the same series of reactions starting with calcium-L-arabonate. Glucose-1-C^{14} was obtained in excellent yields by Isbell's method (15). All three sugars were recrystallized to constant specific activity and were found to have the correct optical rotation.

Fermentations

The organism used was *Aerobacter aerogenes*, strain NRRL 199. It was cultured overnight in a medium containing KH_2PO_4 (0.5%), $(NH_4)_2HPO_4$ (0.4%), $MgSO_4 \cdot 7H_2O$ (0.2%), $FeSO_4 \cdot 7H_2O$ (0.01%), $MnSO_4 \cdot H_2O$ (0.01%), and NaCl (0.01%). The medium also contained 1% of either D-glucose, D-arabinose, or L-arabinose. These sugars were sterilized separately as 3% solutions. The cultures were grown aerobically at 30° C., in 75 ml. of medium in a 500 ml. Erlenmeyer flask, on a rotary shaker (200 r.p.m., $1\frac{1}{4}$ inch eccentricity).

The contents of two to four flasks were combined. The cells were recovered by centrifugation with aseptic precautions, and then resuspended in 12 ml. of a sterile medium containing 2.5% $MgNH_4PO_4 \cdot 6H_2O$, 0.25% K_2SO_4, and 0.002% phenol red. Ten milliliters of this concentrated cell suspension was transferred to the fermentation flask described previously (18). To this was added 5 ml. of a sterile solution containing 0.5 gm. of the 1-C^{14}-labeled sugar. The air was then replaced with purified nitrogen. The temperature of incubation was 32° C. and the pH was controlled manually (18). The pH was maintained between 7–7.5 because a variety of products are formed at this hydrogen ion concentration (20), making it possible to compare the isotope distribution in several different products which were formed simultaneously. In each experiment the labeled sugar was added to cells grown in a medium containing the corresponding unlabeled sugar to ensure adaptation to the sugar in question.

Examination of Fermentation Products

The methods used for the measurement, isolation, and degradation of the fermentation products, and determination of C^{14}, were the same as those of a

previous investigation (18). The ethanol was converted to acetic acid by dichromate oxidation after a chromatographically pure (17) sample had been obtained by distillation through a packed column. In most cases carrier was added at some stage in the isolation or degradation. No activities are reported unless the observed count was twice the background count. It was usually 10 to 100 times this value.

Results

The amounts of the various fermentation products obtained are shown in Table I. The most striking difference between the D- and L-arabinose fermentations was in the amount of 2,3-butanediol formed, more than eight times as much being obtained from L- as from D-arabinose. Other large differences were found for practically all the products. In general, as far as the relative amounts of products are concerned, the L-arabinose fermentation gave results more like the glucose fermentation than like the D-arabinose fermentation.

TABLE I

PRODUCTS OBTAINED BY THE ANAEROBIC DISSIMILATION OF SUGARS BY *A. aerogenes*

Product	Millimoles per 100 millimoles of sugar fermented		
	D-Glucose-1-C^{14}	D-Arabinose-1-C^{14}	L-Arabinose-1-C^{14}
2,3-Butanediol	26.4	2.86	23.3
Ethanol	63.5	52.6	38.0
Acetic acid	33.1	50.0	25.8
Formic acid	52.7	79.6	44.5
Succinic acid	5.95	8.76	4.45
Lactic acid	27.5	3.20	10.8
Propionic acid	Nil	0.46	Nil
Carbon dioxide	97.6	29.1	78.7
Fermentation time (hr.)	12	48	12
Sugar dissimilated (%)	97.2	78.3	94.4
Final cell carbon[1]	11.5	17.0	14.2
Sugar carbon accounted for (%)	92.5	71.3	78.8

[1] *As per cent of initial sugar carbon.*

NOTE: *H_2 not measured so O/R index not calculated.*

A comparison of the distribution of C^{14} in the various fermentation products, however, shows that there was a close relation between the two arabinose fermentations (Table II). No significant differences were found in the intramolecular distribution of C^{14} in any of the fermentation products obtained from D- or L-arabinose. The lactic acid from the pentoses differed from that derived from glucose in the relatively large amount of C^{14} in the carboxyl group. This was also true of formic acid which had the same specific activity as the lactic carboxyl from the same fermentation.

TABLE II

DISTRIBUTION OF C^{14} IN FERMENTATION PRODUCTS

Product	C^{14} concentration as % of that in C-1 of sugar used		
	D-Glucose-1-C^{14}	D-Arabinose-1-C^{14}	L-Arabinose-1-C^{14}
2,3-Butanediol			
CH_3—	38.1	44.0*	36.7
—CHOH—	Nil	Nil	Nil
Ethanol			
CH_3—	40.5	35.2	36.8
—CH_2OH	0.61	0.30	0.48
Acetic acid			
CH_3	41.8	36.1	34.6
—COOH	1.01	0.48	0.34
Lactic acid			
CH_3—	41.8	42.2	41.0
—CHOH—	Nil	Nil	Nil
—COOH	0.57	21.8	23.4
Formic acid	0.70	21.2	22.2
Carbon dioxide	4.57	16.9	20.6
Cell carbon	5.38	6.82	6.88

** High dilution by carrier necessary in this determination reduced precision so this figure is only an approximation.*

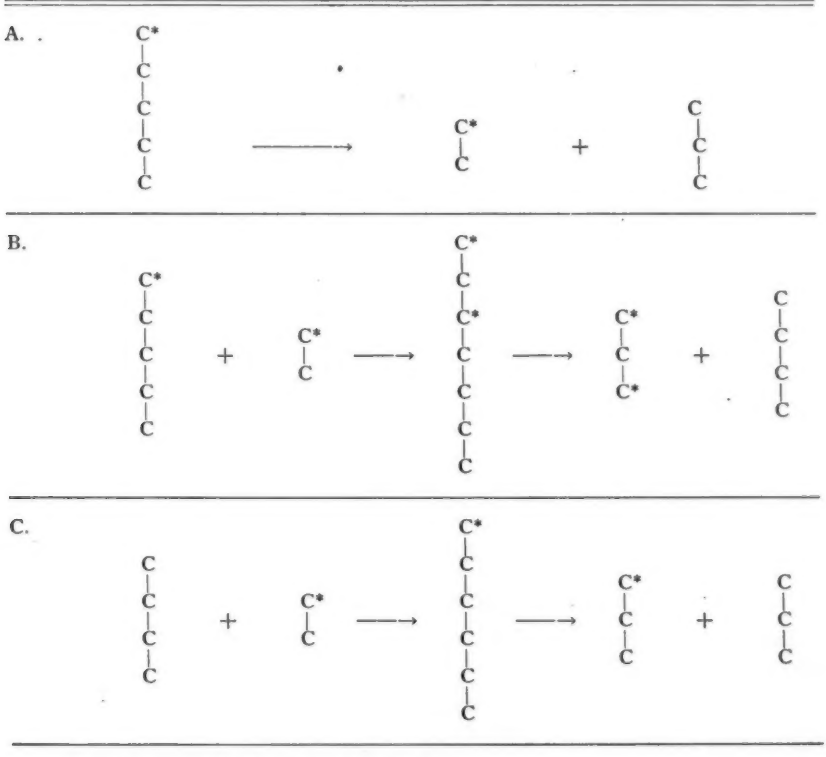
Discussion

The large differences in the proportion of products formed in the D- and L-arabinose fermentations probably are due to a quantitative difference in the enzymic constitution of the cells used. It is a well known fact that differences in environmental factors, particularly pH, during growth result in cells with widely different relative amounts of enzymes. These factors were not closely controlled when the cells used in these experiments were being grown. Since growth in media containing L-arabinose was much more rapid than in media employing D-arabinose as the carbon source the acidity would be expected to increase more in the former and thus give cells with more capacity for 2,3-butanediol formation (20).

The position of the label in the methyl groups of the products obtained from the fermentation of glucose is in agreement with the results obtained with another 2,3-butanediol-forming organism, *Bacillus subtilis* (18). The reactions of the Embden–Meyerhof scheme of glycolysis may play a major role here resulting in the formation of methyl-labeled pyruvate which then gives rise to the products isolated.

To explain the labeling in the products obtained from the pentoses it is necessary to have a mechanism for the formation of both methyl-labeled and carboxyl-labeled pyruvate. Carboxyl-labeled pyruvate has been demonstrated as the intermediate in aerobic dissimilation of glucose by *Pseudomonas saccharophila* (3). The mechanism involved, however, will not explain the anaerobic formation from a pentose, particularly in a system where glucose gives methyl-labeled pyruvate.

It is possible to interpret the data obtained by assuming that the reactions shown in Fig. 1 occur. The pentoses, after phosphorylation and other



* *Denotes atom originally carbon-1 in pentose.*

FIG. 1. Hypothetical C-skeleton changes in pentose metabolism.

changes, undergo fission as shown in Fig. 1A to give a triose (presumably 3-phosphoglyceraldehyde) and an unsymmetrical two-carbon fragment. There is good evidence for this type of fission from work on the dissimilation of pentoses by lactic acid bacteria (2, 7, 5, 21), *Escherichia coli* and yeast (8). The two-carbon fragment may then be used to form a heptulose, as shown in Fig. 1B. Enzyme systems for the conversion of a pentose-phosphate to a heptulose have been demonstrated in animal tissues (10) and spinach (1, 11). The conversion of the heptulose to a mixture of triose and tetrose as shown gives, ultimately, pyruvate labeled equally in the methyl and carboxyl groups. The tetrose does not accumulate and it is assumed that it forms a hexose as shown in Fig. 1C. The hexose then gives rise to methyl labeled pyruvate by the reactions of the Embden–Meyerhof scheme. Evidence for reactions of this general type involving tetroses has been found in enzyme systems obtained from peas (6, 14) and yeast (12). It has been shown that one mole of ribose-5-phosphate and one mole of a heptulose phosphate will react to give two moles of glucose-6-phosphate (16). Also recent work has indicated that L-erythulose is an intermediate in enzymatic conversion by spinach and liver extracts of pentose phosphate to hexose monophosphate (13). These observations can be explained by the reactions in Fig. 1 and thus may be taken as evidence in support of the postulated carbon skeleton changes.

The reactions described explain the results quantitatively as well as qualitatively. If it is assumed that all the pentose is metabolized through the reactions shown in Fig. 1 then three moles of pentose would be converted to five moles of pyruvate. One mole of pyruvate would be labeled in both methyl and carboxyl carbons, one mole in the methyl carbon only and three moles would be unlabeled. This means that on the average the pyruvate methyl carbons would have 40%, and the carboxyl carbons 20%, of the specific activity of carbon-1 of the pentose. This agrees with the results in Table II within the limits of experimental error.

On the basis of this explanation all the pentose is converted to pyruvate which then serves as the source of all fermentation products. Thus the energy-yielding reactions are exactly the same no matter what sugar is being dissimilated since they occur after triose phosphate formation. This fact renders the above hypothesis attractive since it simplifies the problem to that of finding how the various fermentable sugars are converted to triose and avoids the postulation of new types of energy-yielding reactions.

Acknowledgment

The valuable technical assistance of Mr. M. D. Chisholm is gratefully acknowledged.

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THE METABOLISM OF DL-HYDROXYPROLINE-2-C^{14} IN THE RAT[1]

BY R. GIANETTO AND L. P. BOUTHILLIER

Abstract

DL-Hydroxyproline-2-C^{14} was synthesized with an over-all yield of about three per cent as calculated on the amount of carbon dioxide employed as radioactive starting material. DL-Hydroxyproline-2-C^{14} given to three normal rats by intraperitoneal injection was shown to be slowly metabolized. A scheme is suggested for the conversion of this amino acid into glutamic and aspartic acids.

Introduction

Only a limited amount of work has been done to elucidate the metabolism of hydroxyproline in the animal organism and no direct evidence exists as to the nature of the intermediate metabolites which arise from the breakdown of the hydroxyproline molecule. In view of earlier findings (9, 10) that this amino acid is glycogenic, it seems reasonable to assume that hydroxyproline should give rise to either an intermediate of the tricarboxylic acid cycle or to a compound metabolically related to it. Furthermore Weil-Malherbe and Krebs (22) have shown that proline or hydroxyproline when incubated in the presence of kidney slices, in a medium containing ammonium ions, causes an increase of the amide nitrogen, which can be accounted for by the direct formation of glutamine from either one of the two prolines. The same phenomenon was observed when proline or hydroxyproline was replaced by glutamic acid. Perhaps the most interesting contribution has been that of Stetten (18) who has reported that glutamic and aspartic acids had the highest concentration in isotope, among the different amino acids isolated from the body proteins of rats fed N^{15}-DL-hydroxyproline. According to this author, the N^{15} contained in the dicarboxylic amino acids would have derived from the degradation products of hydroxyproline. It was shown by Taggart and Krakaur (19) that the cyclophorase system of enzymes catalyzes the complete oxidation of L-proline to carbon dioxide, water, and ammonia, by way of glutamic acid and the tricarboxylic acid cycle. However under the same experimental conditions, L-hydroxyproline is not completely oxidized. By addition of 2,4-dinitrophenylhydrazine to a portion of the incubation medium, Taggart and Krakaur were able to isolate an osazone corresponding to that of γ-hydroxyglutamic acid semialdehyde. They then concluded that γ-hydroxyglutamic acid might be an intermediate compound in the oxidative breakdown of hydroxyproline.

With a view to gaining further information as to the fate of the carbon skeleton of hydroxyproline in the animal organism, the study of the metabolism of this substance labeled with radiocarbon 14 was undertaken. Since it was

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Contribution from the Department of Biochemistry, University of Montreal, Montreal, Quebec. This paper is from a thesis presented by Robert Gianetto to the Faculty of Science, University of Montreal, 1952, in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Presented in part before the 36th annual conference of the Chemical Institute of Canada, June 4–6, 1953, Windsor, Ontario.

our aim to determine the distribution of C^{14} in the glutamic and aspartic acids isolated from the tissue proteins of the animals under experiment, we decided to label carbon 2 of hydroxyproline. By doing so, we hoped to be able to differentiate, in any one of the dicarboxylic amino acids examined, the C^{14} inevitably introduced by carboxylation reactions from that carried over by the intermediate compounds. The synthesis of DL-hydroxyproline-2-C^{14} was achieved through the reaction scheme illustrated in Fig. 1.

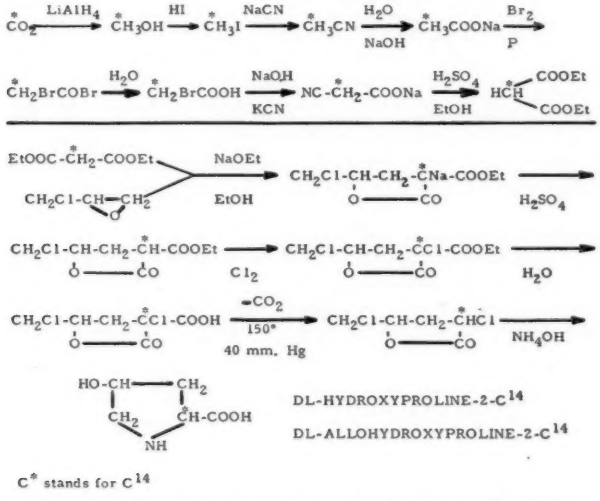

FIG. 1. Scheme for the synthesis of DL-hydroxyproline-2-C^{14}.

Experimental

Synthesis of Labeled DL-*Hydroxyproline*

Sodium acetate.—This substance was prepared by the technique described by Cox, Turner, and Warne (4), with slight modification. Carbon dioxide generated from 13 gm. of barium carbonate by the action of 30% perchloric acid was reduced by a solution of lithium aluminum hydride. The methanol so obtained was refluxed with 75 ml. of hydriodic acid, to give methyl iodide. By reaction of the latter with sodium cyanide, acetonitrile was formed. It was recovered by distillation and hydrolyzed to acetic acid which was removed by steam distillation. The pH of the distillate was adjusted to 8.8 with sodium hydroxide and the solution evaporated to dryness. The residue was then further dehydrated *in vacuo* at about 10 μ pressure and 3.6 gm. of anhydrous sodium acetate was obtained.

Bromoacetic acid.—Anhydrous sodium acetate was transformed into bromoacetic acid by the method of Auwers and Bernhardi (1). The yield of distilled bromoacetic acid was 85% to 90% based on sodium acetate.

Sodium cyanoacetate.—We have modified the method of Scarborough (15) to prepare this substance. Three grams of potassium cyanide was dissolved in a little more than the amount of 3 *M* sodium hydroxide required to neutralize the bromoacetic acid obtained in the preceding step. This solution was added to the bromoacetic acid cooled to 0° C. The reaction mixture was then heated on a water bath for 30 min. and evaporated to dryness under reduced pressure.

Diethyl malonate.—This substance was prepared from sodium cyanoacetate according to the method devised by Christie (3). The yield, based on the amount of carbon dioxide employed as radioactive starting material, was 30 to 35%.

δ-Chloro-γ-valerolactone-α-carboxylic ethyl ester.—The sodio derivative of malonic ester was made to react with epichlorhydrin according to the method of Traube and Lehman (20). The lactone obtained in a 66% yield had a boiling point of 174°–176° C. (corr.) under a pressure of 7–8 mm. of mercury.

α-δ-Dichlorovalerolactone.—This compound was prepared by the method described by Leuchs, Giua, and Brewster (13), with slight modification. The two racemic compounds obtained by chlorination of δ-chloro-γ-valerolactone-α-carboxylic ethyl ester were not separated. The fraction distilling from 149° to 152° C. (corr.) under a pressure of 8–9 mm. of mercury was collected. α-δ-Dichlorovalerolactone was thus obtained with a 75% yield.

DL-*Hydroxyproline.*—α-δ-Dichlorovalerolactone was made to react with ammonium hydroxide according to the method of Leuchs, Giua, and Brewster (13). DL-Hydroxyproline was separated from the DL-allo compound by fractional crystallization of the copper salts, followed by H_2S treatment. Purification of DL-hydroxyproline was achieved by crystallization in water–alcohol. The pure product was obtained with an over-all yield of 3% based on the amount of labeled carbonate used as starting material. Calc. for $C_5H_9O_3N$: N, 10.69. Found 10.80.

Administration of DL-*Hydroxyproline-2-C*^{14} *to Rats*

Doses of the labeled substance were given, by intraperitoneal injection, to three rats fasted for 15 hr. and weighing 180, 162, and 165 gm. respectively. Rat 1 received 65 mgm. of DL-hydroxyproline-2-C^{14} (specific radioactivity 7.4×10^4 counts per minute per mgm.) dissolved in 1 ml. of a phosphate solution buffered at pH 7.4. Rats 2 and 3 received in the same manner 100 mgm. of DL-hydroxyproline-2-C^{14} having a specific radioactivity of 2.44×10^5 counts per minute per mgm. Each animal was placed in a metabolism cage and the respiratory carbon dioxide and urine were collected. After four hours the rats were sacrificed with ether.

Collection of Exhaled Carbon Dioxide and Urine

The respiratory carbon dioxide was absorbed by a sodium hydroxide solution and precipitated as barium carbonate. The urine was collected and the volume completed with water to 100 ml.

Isolation of Urinary Hydroxyproline by the Carrier Method

Six hundred milligrams of DL-hydroxyproline was dissolved in 2 ml. portions of diluted urine and five volumes of cold ethanol were added. The product was recrystallized from water–alcohol to constant radioactivity.

Body Constituents

The carcass of Rat 1 and the entire tissues of Rats 2 and 3 were minced in a Waring blendor in the presence of 10% trichloroacetic acid. The crude proteins were removed by filtration and extracted with 5% trichloroacetic acid, warm acetone, and ether.

The proteins were hydrolyzed with concentrated hydrochloric acid and a number of amino acids were isolated from the hydrolyzates. Glutamic and aspartic acids were isolated as their barium salts which were then decomposed by acidification with sulphuric acid. Glutamic acid was crystallized as the hydrochloride, while aspartic acid was precipitated as its copper salt and regenerated by treatment with hydrogen sulphide. By the method of Stein and Bergmann (17) proline was recovered as the rhodanilate and liberated by reaction of the derivative with pyridine. Nonradioactive hydroxyproline was added to the solution as holdback carrier. Proline was carried down by mixing with an alcoholic solution of cadmium chloride to form an insoluble complex (12) and recrystallized as such. Hydroxyproline was isolated as the reineckate, regenerated by treatment of the salt with pyridine, and purified by crystallization in water–alcohol (11).

Decarboxylation of Glutamic and Aspartic Acids

The glutamic and aspartic acids were decarboxylated by means of ninhydrin according to the method of Van Slyke *et al.* (21) and the carbon dioxide collected as barium carbonate.

Radioactivity Measurements

Known quantities of the substances to be examined were uniformly spread on aluminum cups (7.2 $cm.^2$) and their radioactivity measured with a windowless flow counter. Corrections were made for background and internal absorption.

Results and Discussion

The rate of C^{14} excretion in the respiratory carbon dioxide of each animal is represented in Fig. 2. On the average, about 12% of the injected radiocarbon appeared in the expired carbon dioxide. It is to be noted that the rate of C^{14} excretion observed after the administration of hydroxyproline is much lower than that which has been reported by authors who experimented with rats given C^{14}-labeled amino acids, such as glutamic acid (5), histidine (2, 7, 16), and glycine (2, 8). This fact can be taken as an indication that hydroxyproline is catabolized more slowly than the amino acids mentioned above.

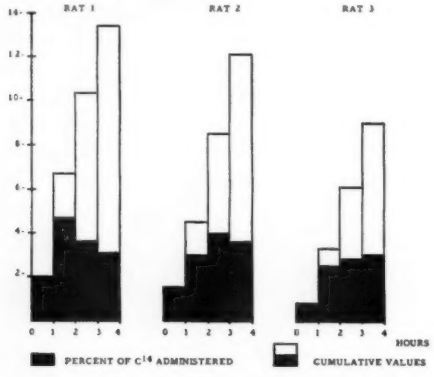

FIG. 2. C^{14} excretion in respiratory CO_2.

The urine of the three rats under experiment contained 20, 25, and 28% respectively of the injected radioactivity. By the use of the carrier technique, we have found that 65 and 63% of the radioactivity present in the urine of Rats 2 and 3 could be accounted for by excreted DL-hydroxyproline.

Among the amino acids isolated from tissue proteins (Table I) glutamic acid had the highest specific radioactivity. Aspartic acid and hydroxyproline contained nearly the same amounts of isotope while proline exhibited very little radioactivity. The radiocarbon contained in the latter amino acid may have originated from its known precursor glutamic acid (6, 14). Since glutamic acid contained more radiocarbon than hydroxyproline, it seems that the

TABLE I

RADIOACTIVITY DATA

Amino acids isolated	Rat 1	Rat 2	Rat 3
	Specific activity, c./min./mM.		
Glutamic acid hydrochloride			
2nd crystallization,	1,720	11,750	10,290
3rd "	1,730	11,010	10,100
4th "	—	11,200	10,100
Hydroxyproline			
2nd "	—	6,950	—
3rd "	—	7,470	—
Aspartic acid			
2nd crystallization,	—	6,790	7,720
3rd "	1,200	6,900	7,600
Proline-$CdCl_2$			
3rd crystallization,	176	1,310	1,460
4th "	—	1,310	1,160

TABLE II

DISTRIBUTION OF C^{14} IN GLUTAMIC AND ASPARTIC ACIDS

Rat No.	Glutamic acid			Aspartic acid		
	Specific activity, c./min./mM.	Specific activity of ninhydrin-liberated CO_2 (alpha-COOH), c./min./mM.	% C^{14} in alpha COOH	Specific activity, c./min./mM.	Specific activity of ninhydrin-liberated CO_2 (both COOH), c./min./mM.	% C^{14} in both COOH
1	1,730	300	17	1,200	630	53
2	11,200	1,150	10	6,900	2,320	34
3	10,100	1,400	14	7,600	1,820	24

injected hydroxyproline was poorly incorporated into the tissue proteins. It is interesting to note that similar results were obtained by Stetten with N^{15}-DL-hydroxyproline (18).

Decarboxylation of glutamic acid by means of ninhydrin showed that only 15% of the radioactive carbon contained in the molecule was present in the α-carboxyl group (Table II). This can be accounted for by carboxylation reactions at the level of the tricarboxylic acid cycle. It is then logical to infer that most of the radioactivity was located in carbon 2. We therefore conclude that hydroxyproline may be converted to glutamic acid in the rat organism.

Samples of aspartic acid isolated from the tissue proteins of the three rats were similarly decarboxylated. We have found (Table II) that 53, 34, and 24% of the total radioactivity of this amino acid was located in either of the two carboxyl groups or possibly in both of them. It should be remembered that the ninhydrin treatment of aspartic acid removes both carboxyl groups.

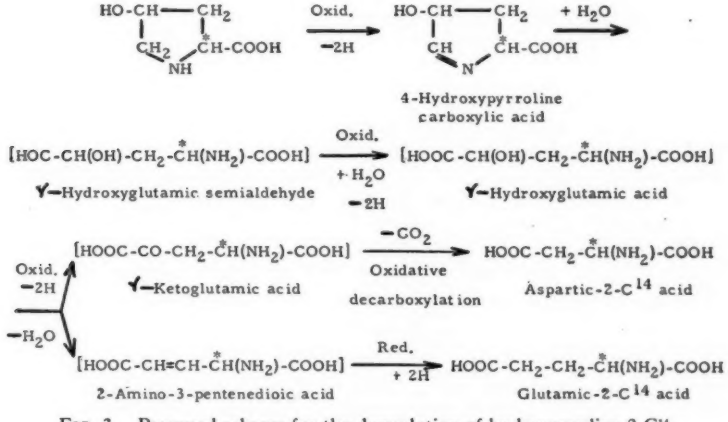

FIG. 3. Proposed scheme for the degradation of hydroxyproline-2-C^{14}.

Therefore the incorporation of the isotope in the carboxyl carbon atoms of aspartic acid most probably occurred through the carboxylation reaction of pyruvate to form oxalacetate, and through the reversible reactions of the tricarboxylic acid cycle. However these metabolic reactions do not explain the presence of C^{14} in the noncarboxylic carbon atoms of aspartic acid. Consequently it seems reasonable to admit that hydroxyproline is also a precursor of aspartic acid *in vivo*.

Assuming that the hypothesis suggested by Taggart and Krakaur (19) is correct, namely that hydroxyproline is metabolized by a pathway analogous to that of proline, we propose the scheme illustrated in Fig. 3 for the conversion of DL-hydroxyproline-2-C^{14} into radioactive glutamic and aspartic acids. All the intermediates are as yet hypothetical.

Acknowledgments

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AUTORADIOGRAPHIC VISUALIZATION OF THE ENTRY AND TRANSIT OF S^{35} IN CARTILAGE, BONE, AND DENTINE OF YOUNG RATS AND THE EFFECT OF HYALURONIDASE *IN VITRO*[1]

By Leonard F. Bélanger

Abstract

The fate of radiosulphate introduced in young suckling rats has been followed by autoradiography of formalin–ethanol fixed celloidin sections. In the cartilaginous epiphyses of long bones, the radioelement has been detected first in the cells, particularly at the hypertrophic level, then in cells and matrix, and finally in matrix exclusively. The incorporated radiosulphate was later displaced towards the ossification area by the growth process. During this migration, the relative intensity of the autoradiograph decreased progressively. This series of events was interpreted as visualization of chondroitin sulphate synthesis, secretion, and utilization or turnover. The S^{35} labelled substance was rapidly hydrolyzed by hyaluronidase except in areas of tendon insertions. In bone and dentine, an over-all initial exchange picture was replaced by a growth line of acid fast, hyaluronidase labile, sulphated material which became displaced along with bone salts by further growth. This material was metachromatic with toluidine blue. It is thought to be also chondroitin sulphate and its possible relationship to mineralization has been discussed.

Introduction

In previous studies by Dziewiatkowski *et al.* (13, 14), Layton (20), and Boström and Odeblad (11), the incorporation in various organs including cartilage of S^{35} administered as sulphate has been demonstrated. Dziewiatkowski (15) has shown the presence in bone of a sulphated compound retained after fixation with formaldehyde saturated with barium hydroxide. The present author (4, 5) has also reported that sulphur labelled compounds can be demonstrated in cartilage as well as in mineralized or demineralized bone, dentine, enamel, and several soft tissues (6) after routine fixation in formaldehyde–ethanol. These localizations were considered to be representative of sulphated polysaccharides or mucoproteins. In the cartilage, Dziewiatkowski (14) and Boström (9) have been able to isolate radioactive chondroitin sulphuric acid and to estimate quantitatively the uptake of ester sulphate by this tissue.

The present autoradiographic studies have been made in order to recognize the site of synthesis of the sulphated substances in cartilage, bone, and dentine; also to follow their fate in these tissues; to try to understand their relationship with mineralization; and finally to determine histochemically, their nature and relative local concentration.

Materials and Techniques

Suckling rats (Sprague-Dawley strain) of four and eight days of age were injected subcutaneously with a single dose of 5 μc. per gm. of separated isotope,

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obtained as weak H_2SO_4. These animals were sacrificed at intervals of one and two hours, one, two, four, and six days thereafter. One half of the skeleton was fixed in a mixture of one part neutral formaldehyde and three parts 95% ethanol (1); the other half was placed for 24 hr. in 4% formaldehyde saturated with barium hydroxide, according to Dziewiatkowski (15). The fixed tissues were dehydrated in ethanol and embedded in low viscosity nitrocellulose (1). Sections of 10–12 μ in thickness were cut. Autoradiographs were made by the modified inversion technique (2), using melted photographic emulsion (7). For purposes of comparison, some sections were demineralized on the slide in formic acid previous to the autoradiographic treatment.

Other sections were incubated from one to six hours at 37° C. in a 0.1% solution of testicular hyaluronidase (Worthington Biochemical Sales Co., Freehold, N.J.) buffered at pH 5.6. Controls were incubated for six hours in the same medium previously boiled for five minutes.

The toluidine blue stain was also applied to some sections in order to detect metachromasia. The results were evaluated by examining the slides in distilled water or in temporary mounts of Crown Oil (Techni Products Co., Buffalo, N.Y.). Permanent preparations were obtained with occasional success, by drying the slides rapidly between fine filter paper, clearing the sections in fresh anhydrous toluene, and mounting them in Permount (Fisher Scientific Co.).

Observations

The following descriptions will be of material fixed in formaldehyde–ethanol. Comparable sections fixed in formaldehyde – barium hydroxide have shown slightly higher Geiger–Müller counts on the average but the autoradiographs revealed numerous spots representing translocations or precipitates

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PLATE I

FIG. 1. A portion of the head of the humerus of a four-day-old rat, sacrificed one hour after a subcutaneous injection of radiosulphate. Inverted autoradiograph, ×32. The hypertrophic cells of the preossifying cartilage show an intense uptake of S^{35}. A lesser synthetic activity is exhibited by the younger cells above.

FIG. 2. The lower extremity of the femur and the patella of a six-day-old rat, sacrificed two days after a subcutaneous injection of radiosulphate. Inverted autoradiograph, ×32. The radioactive material has reached the ground substance where it appears diffuse. There is an area of greater concentration at the level of the hypertrophic cells. The underlying zone of degenerated, mineralized cartilage shows a reaction along the trabeculae, indicating growth transit towards the marrow cavity. There is a weak reaction over the ligaments.

FIG. 3. The upper extremity of the femur of a 10-day-old rat injected six days previously with radiosulphate. Inverted autoradiograph, unstained, ×32. The reaction is now clearly extracellular. The greatest concentration of S^{35} is now visible in the young cells above the hypertrophic zone. The cartilage partitions embedded in the new spicules at the plate retain some radioactivity.

FIG. 4. The articulation of the knee of a six-day-old rat, stained with toluidine blue, ×32. The concentration of metachromatic material in the head of the tibia is seen to increase progressively from the articular surface towards the epiphyseal plate. Localized spots of larger concentration are also visible.

PLATE I

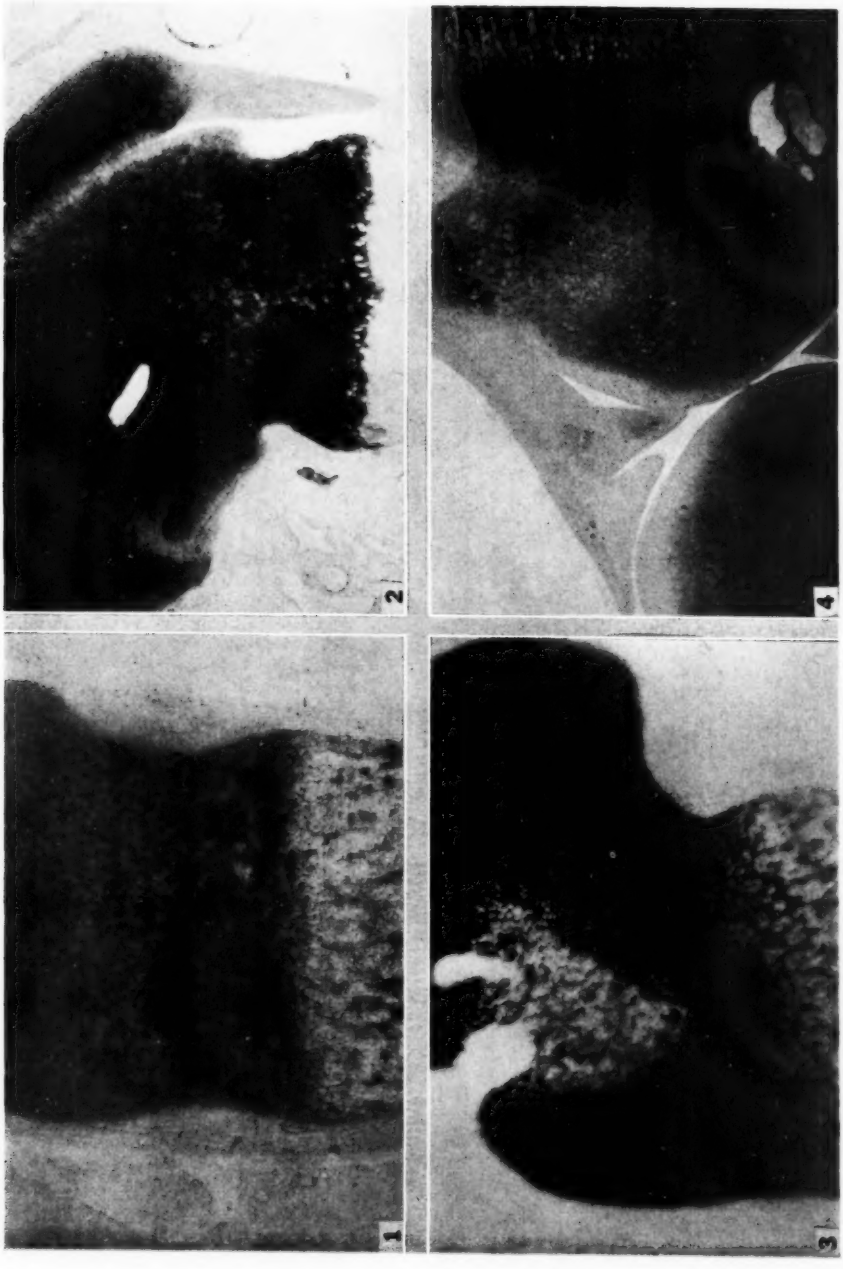
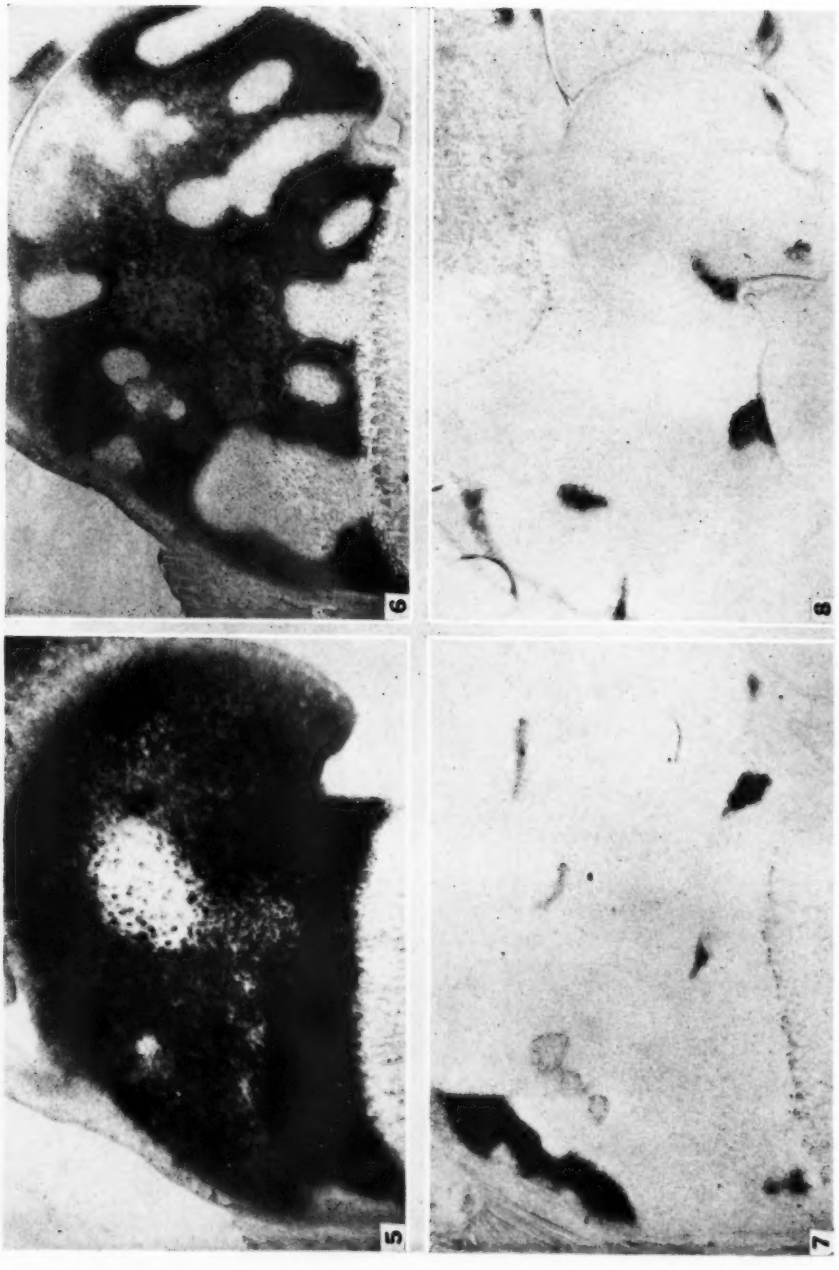
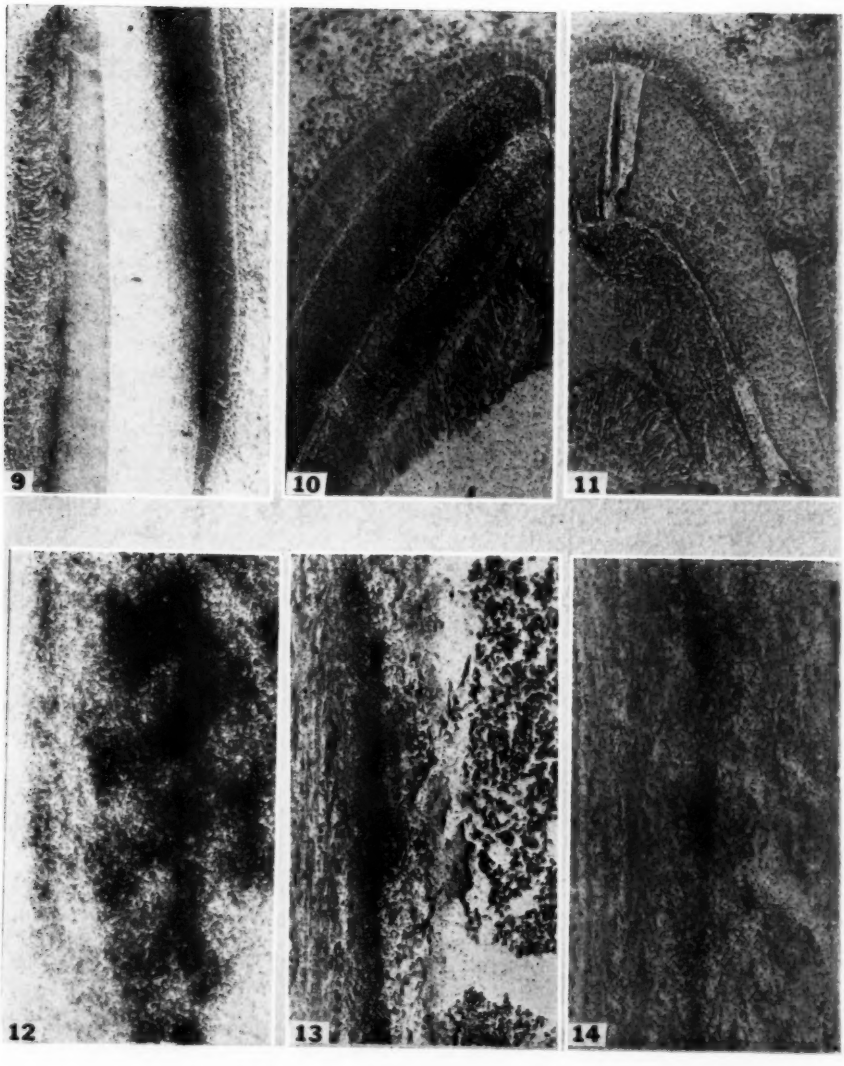


PLATE II

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over the bone marrow and other soft tissues as well as over the skeletal elements. These artefacts, most apparent in the early stages after the introduction of S^{35} and representing possibly some retained mineral sulphates, were disregarded.

Cartilage

In the animals sacrificed one and two hours after the injection of tracer (Fig. 1), the autoradiographs revealed a progressively intense intake by the hypertrophic cells and to a lesser extent by the proliferating cells above. There was a comparable distribution of the intracellular radioactive material throughout the thickness of the cartilage pieces. Autoradiographic examination of the elastic cartilage of the ear has shown that the central larger cells exhibited the greatest relative intake. At two hours there is a minor reaction in the ground substance in the vicinity of the hypertrophic elements (capsule?). At 24 hr. the extracellular radioactivity is more intense and farther reaching. At 48 hr. (Fig. 2), the reaction from the cells and from the ground substance is equally intense. There is still an apparent predominance of radioactivity in the region of hypertrophic cartilage. The presence of S^{35} has become detectable over the trabeculae of calcified cartilage underneath. The tendons, ligaments, and aponeuroses have shown a minor reaction at this stage. The articular surface of the cartilage was only weakly positive.

Six days after the introduction of tracer (Fig. 3), the radioelement has appeared as definitely extracellular in location. By growth, some of the radioactive trabeculae have become embedded in the new bone spicules at the plate which, in autoradiographs of shorter exposure, show a decrease of radioactivity. At this stage, the largest amount of radioactive material seems localized to the zone of proliferating cells, probably because the lacunae are smaller in that area, the result being a relatively larger amount of intercellular material.

Acid demineralization and incubation at 37° C. for six hours after fixation (Fig. 5) did not seem to have any effects on the above mentioned localizations. Hyaluronidase, on the other hand, has hydrolyzed rapidly the sulphur bearing material (Figs. 6, 7, 8). The result of this operation has appeared at first to affect the material irregularly (Fig. 6) but after three hours of treatment,

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PLATE II

Hyaluronidase hydrolysis experiment.

FIG. 5. Upper extremity of the humerus of a nine-day-old rat, injected 24 hr. previously with radiosulphate. Control section, incubated six hours at 37° C. in a boiled solution of hyaluronidase, buffered at pH 5.6. Inverted autoradiograph exposed five days, ×32. The picture is quite comparable to that of an untreated slide.

FIG. 6. Section from the same tissue, incubated one hour in 0.1% hyaluronidase. Patchy areas of hydrolysis are apparent by absence of autoradiograph and basic fuchsin stain. Apparently nonspecific distribution of these areas.

FIGS. 7 and 8. Sections of upper and lower extremity of the humerus from the same specimen. Hyaluronidase, three hours. Inverted autoradiograph, exposed 30 days, ×32. The remaining zones of radioactivity occupy mostly a peripheral location.

some localized remnants (Figs. 7, 8), mostly at the periphery of the cartilage pieces, appeared to have something in common (location, shape, size, relative radioactivity) indicating a possible significance.

Staining of the cartilage with toluidine blue (Fig. 4) shows that there is a progressive increase of metachromatic material from the articular surface towards the zone of hypertrophic cartilage where metachromasia is maximal. Toluidine blue staining of sections incubated in hyaluronidase gives a picture parallel to that of the autoradiographs (Figs. 5, 6, 7, 8). The metachromatic material is retained for the longest time in the ground substance of the hypertrophic cartilage except for small islands of metachromatic material at the periphery corresponding to the autoradiographs in Figs. 7 and 8. Some of these isolated portions of cartilage show, in the histological sections, the presence of enlarged cells and, in places, direct continuity with tracts of fibrocartilage extending out into the ligaments or tendons, so that these represent the intracartilaginous insertions of these tendons or ligaments.

Bone

The bones of the young rats sacrificed one or two hours after the subcutaneous injection of radiosulphur (Fig. 12) have exhibited a generalized picture of S^{35} intake which in the present material appeared proportional to the age of the tissue and consequently to the relative mineralization. Sections of acid demineralized bone of this type (Fig. 13) have revealed that this over-all reaction of the initial phase has disappeared in great part with the minerals and only a band of radioactive material persisted at the diaphyseal zone of growth, immediately under the periosteum.

PLATE III

FIG. 9. Portion of the 1st molar, upper jaw, from an eight-day-old rat, sacrificed two hours after a subcutaneous injection of radiosulphate. Inverted autoradiograph of a decalcified section, ×108. A fine linear reaction is visible at the limit of predentine and dentine. A strong picture is seen at the surface of the enamel with some diffusion inwards. A weaker, diffuse reaction is also visible over the ameloblasts of the growth phase.

FIG. 10. Portion of the 1st molar, upper jaw, from an eight-day-old rat, injected four days previously with radiosulphate. Inverted autoradiograph, ×110. A definite band of radioactive material is visible away from the predentine at a distance inversely proportional to the age of the tissue and followed by a diffuse reaction. In the enamel, the distal mineralized region is most active.

FIG. 11. A portion of the 1st molar, upper jaw, from a 10-day-old rat, injected six days previously with radiosulphate. The radioactive band in dentine has reached the dentino–enamel junction. The reaction in enamel has considerably decreased. Inverted autoradiograph, ×110.

FIG. 12. A portion of the diaphysis of the humerus of a four-day-old rat sacrificed two hours after a subcutaneous injection of radiosulphate. Inverted autoradiograph of a mineralized section, ×110. There is a reaction visible over all parts of the bone. The activity is apparently proportional to the age of the tissue.

FIG. 13. An acid demineralized section of the same tissue as in Fig. 12. Inverted autoradiograph, ×110. Only the reaction at the periphery of the bone persists.

FIG. 14. A portion of the diaphysis of an eight-day-old rat sacrificed four days after a tracer injection of radiosulphate. Inverted autoradiograph of a mineralized section, ×110. The "growth line" seen in Fig. 13 has become displaced inwards by further apposition. The initial over-all exchange reaction seen in Fig. 10 has disappeared.

Four days later, autoradiographs of mineralized sections (Fig. 14) have revealed that only this growth band has persisted and it has become displaced inwards by further apposition of nonradioactive bone substance.

As compared to this sulphur intake at the periosteal border, much less has been revealed in the spicules at the epiphyseal plate. By comparative examination of the spicules along their width, it seemed that the amount of radiosulphur increased from the periphery towards the center, an indication of increasing concentration with maturation.

Staining of the bone with toluidine blue has revealed that all parts of the bone at this stage were metachromatic, particularly when the tissues were examined in water or Crown Oil after staining. This metachromasia was very easily lost in the process of dehydration. With hyaluronidase incubation, it has disappeared rapidly, no trace remaining after the first hour.

Dentine

The entry and fate of S^{35} in enamel has been reported elsewhere (5). The phenomena recorded over dentine were quite comparable to those described in the bone. In the one to two hour stage, an intense over-all reaction indicated the presence of S^{35} in all of the tissue. In demineralized sections (Fig. 9), the generalized autoradiograph has disappeared and only a thin band of radioactive material was recorded at the junction of predentine and dentine.

Four days later, the autoradiographs (Fig. 10) showed that this band had become displaced towards the dentino–enamel junction by further growth and it was followed by a diffuse, weaker image extending to the predentine. The distance between the growth line and the predentine border was proportional to the rate of growth and thus inversely proportional to the age of the tissue.

After six days, the autoradiographic picture of the growth line (Fig. 11) has reached the dentino–enamel junction at the apex of the first molar.

Staining of dentine with toluidine blue has revealed a larger concentration of the metachromatic material than in the bone, indicating that the material is more resistant to dehydration and to hyaluronidase hydrolysis. This substance seemed to increase with the maturation of the tissue at least in tissues of the present age group. After hyaluronidase incubation, the largest amount persisted at the immediate border of the dentinal tubules.

A weak metachromasia was detected in the predentine with a sharp increase at the border of predentine and dentine. A diffuse metachromasia was also seen in the dental pulp with areas of concentration at the apex of the pulp and also as large transverse bands at the entrance of the pulp chamber, this last occurrence being most apparent in the incisor.

Discussion

Cartilage

This autoradiographic study after administration of radiosulphate indicates first that the sulphate ions diffuse rapidly through the substance of the

cartilage and are picked up by the cells and incorporated into an organic molecule which is retained by the present method of fixation and histological treatment (Fig. 1). This substance is believed to be chondroitin sulphate. In the area of degenerated cartilage where the cells are dead or dying, no autoradiograph has been recorded. The origin of chondroitin has never been clearly established. Sylven (27) states that it is usually believed that chondroitin sulphuric acid is synthetized by the chondrocytes but that proof is lacking. Clark and Clark (12), on the other hand, suggest that it might be formed in the intercellular substance. Apart from the autoradiographic demonstration of the presence of S^{35} in the chondrocytes, our material stained with toluidine blue has revealed some metachromatic substance within the cells and also within the lacunae particularly in the region of hypertrophic cartilage. Furthermore, Greulich and Leblond (17) have recently published a picture of autoradiographic entry of C^{14} in cartilage which has a striking resemblance to Fig. 1 of the present work. They suggest that this is representative of "carbohydrates, proteins or complexes of these two types of substances." These combined facts indicate that the early autoradiographs demonstrate the synthesis of a sulphated polysaccharide complex by the chondrocytes, particularly in the area of hypertrophic cartilage. In another recent study (9), Boström has recorded the entry of S^{35} into the cartilage by measuring, at various time intervals after the administration of tracer, the specific activity of the tissue. This work has revealed that the radioactivity of the ester sulphate is maximal at 30 hr. and decreases slowly thereafter. The author concludes that the early high intake must be limited to the sulphate exchange of the chondroitin sulphuric acid. If such were the case, it would seem that this exchange would be proportional to the tissue content of chondroitin which is much greater in the ground substance than in the cells, as demonstrated by various means, and consequently a reversal of the present autoradiographic picture would be expected.

Subsequent autoradiographs of the present series reveal that synthesis of the tagged sulphated compound takes place during the first 48 hr. (Fig. 2); secretion begins very early and at the end of two days the intercellular content of fixed radiosulphur is apparently equivalent to that of the cells. The larger rate of incorporation at the level of the hypertrophic elements already recognized by Dziewiatkowski (15) results in a larger proportional content of sulphated polysaccharide in the ground substance at that level (Figs. 2 and 3). Six days after the initial intake (Fig. 6) we find that the cellular elements are free of sulphated compound which is now present in the matrix exclusively. The curve of S^{35} content of the blood published by Boström (9) indicates a peak of activity at 10 hr. and a rapid decrease during the next 30 hr. whereby most of the radiosulphur would be outside the blood at the end of 40 hr. These facts are well in accord with the present findings. On account of the presence of enlarged lacunae in the hypertrophic cartilage and in the degenerated calcified cartilage, where the radioactive trabeculae have now migrated by the process of growth, it seems that these regions contain less of

the radiosulphur than the younger cartilage above, in which the rate of synthesis was slower but where also the honeycomb is finer, producing a greater matrix cell ratio.

The relative intensity of the autoradiographs developed at various intervals reveals that there has been no great accumulation of S^{35} in the intercellular material originally at the hypertrophic level, indicating a probable rapid turnover and utilization.

The cartilage trabeculae incorporated in the new bone spicules at the epiphyseal plate appear to be less radioactive than the nonincorporated ones above as demonstrated by autoradiographs of relatively short exposure. This would indicate the possibility of a certain amount of decrease and maybe utilization at the contact of the bone. Nevertheless autoradiographs of long exposures show that some of the radioactive material persists in the bone spicules at six days down to the free end tip where resorption takes place.

The S^{35} tagged material of the cartilage has proved resistant to acidity and heat (Fig. 5) but it has rapidly disintegrated in the presence of hyaluronidase (Fig. 6) except for some apparently specific locations at the periphery of the cartilage pieces where tendons and ligaments were found inserted (Figs. 6, 7, and 8). It is impossible to state from the present series of experiments whether the substance responsible for these localized remnants of radioactivity represent higher concentrations of the general chondroitin sulphate A or whether they would be islands of chondroitin sulphate B, which Meyer and Rapport (23) have discovered in heart valves, tendons, and aortas and which has been found by them to be hyaluronidase resistant.

Bone and Dentine

For purposes of comparison, bone and dentine will be discussed together. In the transactions of the 4th Conference on Metabolic Interrelations, Follis (16) states that "the polysaccharide component of bone has not been studied at all extensively; that is we do not know whether there is chondroitin sulfuric acid in bone." Elsewhere in his paper, this author states that bone is metachromatic. Wislocki and Sognnaes (28) have described metachromatic material in dentine. Johnson and Bevelander (18) have also told of metachromasia of the dental pulp and uncalcified dentine which disappears as calcification proceeds. On the other hand, Dziewiatkowski (15) has recognized the entry of S^{35} in bone wondering whether "there is any relationship between the metabolism of sulfate sulfur and phosphorous in loci where ossification is progressing".

The present work has demonstrated that in the few hours after its administration, the radiosulphur is found largely deposited over all the mineralized bone and dentine. In our young material (Fig. 12), the intensity of the sulphur autoradiograph is apparently proportional to the degree of mineralization. This large over-all reaction is comparable to that which was obtained

with P^{32} when, in some previous work (22, 3), the entry and fate of radiophosphate was studied. This is interpreted as representing atomic interexchange between the liquid phase of the tissue fluid and the solid phase of bone or dentine. The atoms of S^{35} responsible for this physical phenomenon are apparently attached to the mineral portion of the bone and dentine or at least they are removed with the mineral constituents of these tissues, by the action of acids (Fig. 13). Whether these atoms belong to mineral sulphates or enter into some other combination, it is presently impossible to tell.

However, after acid demineralization, a thin band of radioactive material persists in bone (Fig. 13) and dentine (Fig. 9). With time, the relative position of this band is altered by further appositional growth of tissue, and the autoradiographic picture appears to move away from the formative vicinity of the osteoblasts (Fig. 14) and the odontoblasts (Figs. 10 and 11). This band is interpreted as representing a sulphated polysaccharide, probably chondroitin sulphate, which appears in the ground substance of osteoid and predentine and becomes intimately concerned with the phenomenon of mineralization since its behavior is from then on parallel to that of P^{32} and Ca^{45} as they enter into the mineral components of bone (22) and teeth (3, 8, 19, 21).

This material has stained metachromatically with toluidine blue, more intensely in dentine than in bone. As compared to that of cartilage, it has been more rapidly hydrolyzed by hyaluronidase. This would relate it to chondroitin sulphate type "A", of Meyer and Rapport (23).

It seems unlikely that any of the radiosulphate demonstrated by autoradiography, in the course of the present experiments, would have become incorporated into sulphoproteins. The epidermis and hair proper, which contain large amounts of these substances, have been consistently negative in the autoradiographs (6). On the other hand, in a previous analysis, Boström and Gordell (10) have reported that very little sulphate is utilized in the synthesis of the sulphur containing amino acids, taurine, cystine, and methionine.

The intimate relationship of the sulphopolysaccharides with the onset of mineralization recalls the theory of the "local factor" of calcification expressed by Sobel (25) whereby according to this author (26) and to Newman, Boyd, and Feldman (24), chondroitin sulphate would be a cation-binding agent, retaining calcium in preparation for its subsequent release and more stable precipitation in the presence of an abundance of phosphate ions.

Acknowledgments

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THE BIOSYNTHESIS OF CELL WALL CARBOHYDRATES

GLUCOSE-C^{14} AS A CELLULOSE PRECURSOR IN WHEAT PLANTS[1]

By Stewart A. Brown and A. C. Neish

Abstract

The formation of cellulose in wheat plants has been studied using as precursors glucose-1-C^{14}, sorbitol-1-C^{14}, and succinic acid-2,3-C^{14}, which were administered to the plant aseptically by injection of sterile solutions into the hollow internodes. Glucose obtained by hydrolysis of the cellulose, isolated after the plants had grown for 24 to 48 hr. following the injection of labelled glucose, showed a concentration of at least 50% of the total C^{14} in carbon-1. When sorbitol was injected most of the carbon-14 was fairly evenly divided between C-1 and C-6, and when succinic acid was used evidence of a comparatively uniform labelling of the glucose was obtained. The results indicate that there is considerable conversion of glucose to cellulose without skeletal rearrangement, and that there is also appreciable cellulose formation from resynthesized glucose. Sorbitol is not an intermediate between glucose and cellulose; it is suggested that it is converted to glucose largely by way of three-carbon fragments.

Introduction

Much has been learned in recent years about the mechanism of formation of a number of polysaccharides, among which starch and glycogen serve as prominent examples (8). In comparison, however, the chemistry involved in the formation of cellulose has been the subject of very little investigation, a rather surprising fact in view of the fundamental importance of this polysaccharide in higher plants. Until quite recently, although it has been generally assumed that cellulose arises in some way through the polymerization of glucose, experimental evidence in support of this theory has been meager. Several years ago Tarr and Hibbert (19), Hestrin and co-workers (9), and Kaushal and Walker (12) demonstrated a synthesis of cellulose membranes by *Acetobacter* in the presence of a number of carbohydrates and other compounds, including glucose, but no evidence was presented to show that glucose possessed any special function as a cellulose precursor. Subsequently, Greathouse and co-workers (6) further investigated this problem using C^{14}-labelled carbohydrate intermediates, but unfortunately complete details of this investigation appear not to have been published as yet.

While the present studies were in progress, Greathouse (5) reported the first experimental evidence for the direct utilization of glucose in cellulose formation by a higher plant. After administering D-glucose-1-C^{14} to cotton bolls during the period of most rapid cellulose synthesis he found that within experimental error all the C^{14} was present in the C-1 position of the glucose units of the cellulose.

It was noted, during studies in this laboratory on the formation of lignin in wheat plants, that after the degradation of the lignin by heating the cell

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wall material with nitrobenzene and alkali, a residue of crude but easily purified cellulose remained (2). Following the administration of D-glucose-1-C^{14} to the plants it was found that this cellulose contained appreciable radioactivity, and on degradation of the cellulose it was found that the constituent glucose retained a concentration of C^{14} in the C-1 position, suggesting a direct polymerization of the administered glucose. The conversion of labelled glucose to cellulose has been studied further using varying periods of administration, and in addition the function of the metabolically related compounds sorbitol and succinic acid as cellulose precursors has been investigated.

Experimental

Cultivation of the Plants

The Thatcher wheat plants used in the four-day activation experiments were grown in the artificial nutrient medium previously described (2). In the 24- and 48-hr. runs conducted at a later date plants grown in this way were no longer available, and wheat grown in potted soil was used instead. In all cases plants grown 62 days from seeding, and kept in the dark at least 24 hr. before administration of the carbon-14, were used. It is convenient to use enough plants to obtain about 5 gm. of dry material.

Synthesis of C^{14}-Labelled Compounds

The glucose-1-C^{14} was prepared by a cyanhydrin synthesis (10) with cyanide-C^{14} serving as the source of carbon-1.

Succinic acid-2,3-C^{14} was synthesized from 1,2-dibromoethane-1,2-C^{14} (3) by condensation with inactive potassium cyanide and hydrolysis of the resulting nitrile.[2]

Sorbitol-1-C^{14} was prepared by hydrogenation, in the presence of Raney nickel, of the mother liquors from the crystallization of glucose-1-C^{14}. These liquors already contained an appreciable quantity of sorbitol formed in the amalgam reduction of the D-glucono-δ-lactone. The sorbitol was purified as the pyridine complex, which was recrystallized to constant specific radioactivity. After decomposition of the complex, the free sorbitol was again recrystallized to constant specific activity.

Preparation and Administration of Radioactive Solutions

The radioactive compounds were weighed into 10-ml. serum bottles and dissolved in sufficient water to make one-half molar solutions. Rubber caps were placed on the bottles, and the whole was autoclaved for 15 min. at 120° C.

The sterile solutions were administered to the plant by injection into the hollow internodes of the stem. The glass jar (from which the nutrient solution had been temporarily removed) or the flowerpot, as the case may be, was supported on an iron ring attached to a stand so that the plant hung in an

[2] *The authors are indebted to Dr. J. E. Stone, formerly of this laboratory, for making available a supply of labelled succinic acid.*

inverted position. Areas at either end of the internode to be injected were then swabbed with 70% ethanol, and the point of a sterile dissecting needle was inserted into the lower[3] end to allow air to escape during the injection. A 0.5 ml. aliquot of the desired solution was drawn into a sterile 1 ml. syringe by way of a No. 27 needle inserted through the serum bottle cap. The needle was then inserted, at a small angle to the stem, into the upper end of the internode, and 0.1–0.2 ml. of solution was injected. The lower puncture was sealed with a drop of flexible collodion, and after withdrawal of the needle the upper puncture was sealed in the same way. The procedure was repeated at each of several internodes until 0.5 ml. had been injected into each plant. When the plant was returned to an upright position, the solution remained in the upper part of the internode, being held there by surface tension, and leakage by gravity through possible imperfect seals was very rarely encountered.

With a little practice, two people working together can inject a series of plants in a short time with little difficulty. The two critical points which must be observed to avoid loss of solution are (i) ensuring an adequate puncture for air displacement, and (ii) holding the flat side of the needle tip at right angles to the stem while the latter is being pierced. The technique has been proved aseptic by taking swabs from within the stem after harvesting, when the injected solution had been absorbed. The procedure makes easy the administration of numerous compounds to wheat plants grown under normal conditions without the complications of bacterial action; presumably it is also applicable to other species with hollow stems.

Isolation of Cellulose

After harvesting, the plants were dried in a 60° circulating oven overnight. The heads and roots were removed and the remaining material was cut into small pieces and ground to pass a 60-mesh screen. The ground material was extracted in a Soxhlet extractor with ethanol–benzene, 1 : 2, until the solvent was colorless; it was then dried and further extracted with hot water. The insoluble residue was oxidized with nitrobenzene in alkali, and the cellulose remaining was isolated and purified, as described in a previous paper (2).

Degradation of Cellulose

A sample of the purified cellulose, weighing 1 to 1.5 gm., was hydrolyzed by treatment with 72% sulphuric acid at room temperature for one week; it was then boiled with the diluted acid for 15 hr. (13). After removal of the sulphuric acid as barium sulphate and concentration of the filtrate to about 25 ml., the latter was stirred separately for one-half hour with Amberlite IR-100-H cation exchange resin, and Amberlite IR-4B or Duolite A-4 anion exchange resin, in a beaker. Any slight color remaining after these treatments was easily removed by brief warming with decolorizing charcoal. The filtrate from this treatment was concentrated in a current of air to heavy sirup, which crystallized readily after it had been seeded and refrigerated overnight.

[3] *"Lower" in this discussion means toward the root of the plant, and "upper", toward the head.*

A sample of this glucose was chromatographed on Whatman No. 1 paper, the upper layer of a butanol – acetic acid – water mixture in the ratio of 4 : 1 : 5 being used as developing solvent (16). On spraying with aniline phthalate reagent (17) a strong spot was observed having an R_f value equal to that of a reference glucose spot. A second faint spot corresponding to cellobiose was also visible, indicating that hydrolysis of the cellulose to glucose was not quite quantitative.

Carbon-1 was isolated as formate from 100 mgm. of this glucose by heating with 10% hydrobromic acid according to the modification of Sowden's procedure (18) described by Abraham, Chaikoff, and Hassid (1). No attempt was made to remove the small amount of cellobiose beforehand, as treatment of a pure sample of this disaccharide with 10% hydrobromic acid resulted in formate yields identical to those obtained with glucose. The formate, in about 50 ml. of water, was oxidized to carbon dioxide by being heated for 1.5 hr. with 3 gm. of mercuric oxide in the presence of 5 ml. of sirupy phosphoric acid. The carbon dioxide was swept out in a current of CO_2-free air, trapped in sodium hydroxide solution, and precipitated as barium carbonate in the usual way.

The remainder of the glucose (700–1000 mgm.) was converted to methyl-D-glucoside by the method of Fischer (4). The glucose, dried *in vacuo* at 70° in a reaction tube, was refluxed for one-half hour with 10 ml. of 0.25% methanolic hydrogen chloride. The tube was then sealed and heated in an oven at 100° for 50 hr. The solution was decolorized with charcoal and concentrated to a sirup. On dilution with about 5 ml. of absolute ethanol and stirring, crystallization of methyl-α-D-glucoside began almost at once, and after several hours' refrigeration the crystals were filtered off. Two recrystallizations from ethanol were sufficient to bring the product to a constant specific rotation $[\alpha]_D^{20} = 159.6$, and constant specific activity.

A sample of the pure methyl-α-D-glucoside (0.5–1.0 mM.) was subjected to the periodate oxidation of Jackson and Hudson (11), and carbon-3 was recovered as strontium formate. This was purified by steam distillation as formic acid, which was then oxidized to carbon dioxide as described above and finally isolated as barium carbonate. The other product, D'-methoxy-D-hydroxymethyl-diglycolic aldehyde, after removal of the ethanol, was taken up in 20 ml. of 0.2 *N* sulphuric acid, and treated with 3 to 4 ml. of 0.4 *M* periodic acid. After standing for one hour at room temperature the solution was heated for an additional hour on the steam bath, and the formaldehyde representing C-6 was distilled, along with about 60 ml. of water, into a 1% solution of dimedon in phosphate buffer. The formaldimedon was plated by filtration and counted.

Determination of Radioactivity

Radioactivity of all samples was measured and calculated as previously described (2). A Marconi type 138–660 and a Nuclear Measurements Corp. PC-1 proportional scaler were used.

Results and Discussion

A rather surprising and especially worth-while aspect of the injection technique used in this investigation is the ability of the plant to absorb, without apparent ill effect, relatively enormous amounts of compounds which are toxic when administered in other ways. Newton and co-workers (15), for example, have reported that certain substituted phenols, including vanillin, are toxic when concentrations of the order of 300 p.p.m. are injected into wheat leaves. In contrast, we have injected a solution of vanillin (as the sodium salt) having a concentration of 50 times the above figure into the internodes; this solution was absorbed by the plant, and no gross symptoms of toxicity were exhibited over a period of several days. The explanation for this difference appears to lie in the relative slowness of entry into the vascular system through the stem.

Following the administration of glucose-1-C^{14} to a growing plant, the distribution of carbon-14 among the carbons of cellulose will be dependent on the relative rates of those reactions which compete for the available glucose. If conditions are such that cellulose synthesis is proceeding at a very high rate, and if glucose is indeed a close precursor, it will be theoretically possible for all the administered glucose to undergo conversion to cellulose; in this case the labelling in the cellulose will be confined entirely to the carbon-1 of glucose. On the other hand, if cellulose synthesis is not rapid enough to utilize all the available glucose, some of this labelled glucose will undergo breakdown and resynthesis. The GREATER THE PROPORTION of cellulose formed from resynthesized glucose, the greater will be the tendency toward a uniform labelling of the cellulose. Other things being equal, it can be assumed that, if glucose is a close precursor of cellulose, the rate of its conversion to the polysaccharide will be reflected in the concentration of C^{14} in the carbon-1 position. By analogy to mechanisms known to be involved in the biosynthesis of other polysaccharides (8), it is probable that any reactions of glucose leading to cellulose would occur only after phosphorylation.

TABLE I

INCORPORATION OF C^{14} INTO WHEAT STRAW CELLULOSE FROM LABELLED COMPOUNDS

Expt. No.	Compound administered to plants	C^{14} content of administered compound, μc./mM.	Activation time, hr.	C^{14} in glucose from cellulose, μc./mM. $\times 10^4$			
				Total	C-1	C-3	C-6
1	Glucose-1-C^{14}	3.7	96	177	78.6 (44%)	7.0	20.2
	Succinic acid-2,3-C^{14}	3.0	96	178	21.3 (12%)	42.2	26.0 (14.5%)
2	Glucose-1-C^{14}	3.7	24	21	15.7 (75%)	—	—
		15.0	24	270	136.0 (50%)	9.2	42.9
3	Glucose-1-C^{14}	4.4	48	89	54.0 (61%)	—	—
	Sorbitol-1-C^{14}	4.4	48	20 (21*)	10.0 (50%)	2.0	7.8 (38%)

* *Corrected for dry weight of plants.*

Table I summarizes the results of several experiments involving a study of glucose and two metabolically related compounds, sorbitol and succinic acid, as cellulose precursors. The plants injected in experiment No. 1 were allowed to grow subsequently for 96 hr. Similar amounts of C^{14} were given, but the runs with glucose and succinic acid were carried out at different times on different groups of plants, so that a quantitative comparison of the two sets of results should not be made. In the case of succinate, as expected, the three carbons isolated show a fair uniformity of labelling; they do not differ by more than a factor of two, and together account for just half of the total carbon-14. The high concentration in C-3, which would not be expected on the basis of known metabolic pathways, requires further study. The glucose-feeding experiment, however, resulted in the isolation of 44% of the C^{14} in C-1. The other two carbons isolated possessed much less activity, but sufficient to render unlikely the possibility that a concentration approximating that in C-1 was present in any other single carbon atom.

Shortening the growing time after injection of the radioactive glucose to 48 and 24 hr. resulted in a somewhat higher fraction of the total carbon-14 being recovered in C-1. As shown in rows 2 and 3 of Table I, from 50 to 75% resided in this position. The accuracy of the latter figure is probably not high, as for some unknown reason total activity in this experiment was low, but it can be stated that when the growing time does not exceed two days following activation at least half of the activity in the glucose molecule is concentrated in carbon-1.

The possibility that glucose is converted before condensation into a related six-carbon compound without skeletal rearrangement prompted the investigation of sorbitol as a cellulose precursor. This hexitol is found in several plants, and because of its close configurational relationship to glucose appeared to be a conceivable alternative, although little is known about its metabolism in higher plants. Identical molar quantities of glucose and sorbitol, containing identical amounts of C^{14}, were injected into two groups of plants grown under the same conditions at the same time. Each group was worked up in the same way, and the results in the two cases are compared in row 3. The bracketed value for the total "sorbitol" carbon-14 is corrected for the slightly greater dilution occasioned by a somewhat larger amount of dry plant material. It can be seen that sorbitol is less than one-quarter as efficient a cellulose precursor as glucose, and therefore cannot be an intermediate between glucose and cellulose. An unexpected result is the similar concentrations of carbon-14 at C-1 and C-6. Here again the low total activity precludes high accuracy, but it may be tentatively concluded that a large percentage if not all of the glucose formed from sorbitol is resynthesized from 3-carbon fragments. Why the plant should not be capable of a more direct conversion is not immediately evident.

It is perhaps not surprising that the almost complete retention in the original position of the C^{14} administered as glucose, and the high percentage

of glucose converted to cellulose in the cotton bolls studied by Greathouse (5) have not been observed in wheat. Glucose administered under such conditions that it is transported almost immediately to the cotton boll at the time of most rapid cellulose formation is entering an organ largely composed of tissue specialized for cellulose synthesis, and a very large percentage of the glucose available at this site must be utilized in this way. Any glucose that is not so utilized is apparently converted into other stable compounds and so escapes degradation. Such conditions may be assumed to be exceptional among higher plants. As far as the authors are aware, the optimum conditions and period for cellulose formation in wheat have not been determined, and while it may be possible to attain larger direct conversion than that found in the present work, it is doubtful whether the values would ever approach closely those obtained on cotton bolls.

There is at present little evidence on which to base theories about the reactions leading from glucose to cellulose. Very recently, Mortimer (14) has found that although the cellulose (or a closely-related polyglucosan) of sugar beet leaves acquires carbon-14 during 10 sec. exposure to $C^{14}O_2$, free glucose does not become active within this period. It seems established, then, that free glucose at least is not a cellulose precursor during normal plant metabolism, and this supports the idea that in the present work and that of Greathouse (5) the administered glucose-C^{14} must have been phosphorylated before being incorporated into cellulose. Beyond this it is possible to do little more than speculate, and any scheme put forth now must be based on analogy to reactions established for the formation of other polyglucosans. Two such mechanisms may be considered as most probable. The first is the direct condensation of glucose phosphate, a process already demonstrated in starch synthesis (8), and the second is a transglycosidation from sucrose, such as has been shown to be involved in dextran formation by *Leuconostoc* (7). Both the reactions used in the above examples yield α-linkages, of course, so in either type of reaction it is necessary to postulate an enzyme system capable of forming β-linkages to account for cellulose formation. It is felt that an extension of the present techniques to a study of sucrose as a possible cellulose precursor might yield data of considerable interest.

Because of the strong evidence for the formation of cellulose from glucose in two such different genera as *Triticum* and *Gossypium* among the higher plants, as well as in *Acetobacter*, it can be inferred that the reaction is a general one. This should facilitate subsequent studies on the conversion of various labelled compounds to glucose in the plant as this sugar, once formed, is fixed in large amounts in an easily isolated state, whereas the isolation of free glucose in most plants presents considerable difficulties.

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ESTIMATION OF PENTOSE NUCLEIC ACID AND DESOXYPENTOSE NUCLEIC ACID IN THE LIVER AND BRAIN TISSUE OF MICE FOLLOWING THE FEEDING OF THE INSECTICIDE ALDRIN (HEXACHLOROHEXAHYDRODIMETHANONAPHTHALENE)[1]

By E. Annau

Abstract

Aldrin fed to mice in sublethal concentrations induced, after a period of time, increase in DNA content of the liver tissue, while its PNA content remained essentially unaltered. A slight increase in the weight of the brain was noted, which did not affect the DNA or PNA content of the brain tissue.

Introduction

The DNA content of normal somatic tissue is generally constant while proliferative or embryonic tissue shows an increase in this nucleic acid (4). Results of estimations for PNA in various tissues under different experimental conditions are contradictory and at the present time a consistent relationship between this cytoplasmic component and cell activity has not been found, although efforts have been made to correlate protein synthesis and PNA production (4). In previous communications (1, 2) it has been reported that prolonged feeding of the highly chlorinated insecticide aldrin (hexachlorohexahydrodimethanonaphthalene) to mice induces a considerable increase in the liver parenchyma. An attempt has been made in the present study to estimate the DNA and PNA in the liver and brain tissue of mice following feeding of aldrin.

Materials and Methods

Nucleic Acid Determination in Mouse Liver

For the purpose of this experiment, male albino mice, derived from this Institute's stock colony and ranging in weight from 25 to 30 gm., were used exclusively. As previously reported (1, 2) all animals were fed on ground Master Fox Breeder Starter laboratory chow. Crystalline aldrin, in doses of 25, 30, 40, and 50 mgm., dissolved in 10 ml. corn oil, was mixed with 1 kgm. of food for the experimental animals; in this way final concentrations of 25, 30, 40, and 50 p.p.m. were attained, as indicated in Tables I and II.

Mice were killed by decapitation at various time intervals during the experimental period. The organs were removed rapidly, weighed, and analyzed. Carcass weight and the liver to body ratio were determined as previously described (1, 2). To estimate water content, weighed samples of liver tissue were dried for 18 hr. at 103° C.

The nucleic acid content of the tissues was estimated by using a modified method of Schmidt and Thannhauser (15). A weighed amount of liver tissue, approximately 1 gm., was ground with chilled sand in a cold mortar and

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suspended in 20 volumes of ice-cold 7% trichloroacetic acid. The suspension was stirred mechanically for 15 min. after which an equal volume of absolute ethanol was added and stirred for an additional five minutes at a temperature of 0° C. The suspension was then filtered through Whatman No. 40 filter paper, on which a piece of washed linen was placed. This method facilitated filtration.

The residue was washed with ice-cold 1% trichloroacetic acid, followed by one washing with distilled water and one with 2% sodium bicarbonate solution in order to neutralize the remaining acids. The residue was dried by subsequent washings with absolute ethanol and ether. Air was drawn through the filter for several minutes to dry the residue completely. The residue was removed from the filter, scraped off the linen cloth, and finely ground in a mortar and resuspended in a 3 : 1 ethanol–ether mixture (about 20 to 30 ml.). The suspension was boiled for 10 min.; it was then centrifuged and suspended again in the ethanol–ether mixture and boiled for five minutes, centrifuged, and washed with ether. The sediment was resuspended in 50 ml. of a 1 : 1 methanol–chloroform mixture and boiled for 30 min., cooled and filtered through Whatman No. 40 filter paper, and washed liberally with ether. It was dried as before, removed from the filter paper, and ground again to a fine powder in a mortar. The powder was transferred into a tube and thoroughly mixed with 0.5 *N* potassium hydroxide solution. After the tube was closed, the suspension was incubated for 18 hr. at 38° C. During the incubation period most of the suspension dissolved. The insoluble fraction was separated by centrifuging at high speed. Acid fractionation and chemical analyses were carried out as indicated by Schmidt and Thannhauser (15). The method of Delory (6) was used for the precipitation of inorganic phosphorus, which was determined by the method of Fiske and Subbarow (8).

Nucleic Acid Estimation in Mouse Brain

Freshly prepared and weighed brains of two mice were homogenized for approximately four minutes in a Potter–Elvehjem homogenizer at 0° C. with 4 ml. of ice-cold distilled water. Six milliliters of ice-cold acetone was added to the homogenate and stirred for 15 min. in the cold. The suspension which formed after the addition of acetone was centrifuged for five minutes and the supernatant removed. The sediment was washed subsequently with a 3 : 2 acetone–water mixture and with pure acetone. After centrifuging, the sediment was resuspended in a 3 : 1 ethanol–ether mixture, boiled for five minutes, recentrifuged, and the supernatant discarded. The sediment was extracted in a heated water bath for 20 min. with 20 ml. of a 1 : 1 methanol–chloroform mixture, and after cooling, collected by centrifuging. A final washing with absolute ether removed any remaining methanol–chloroform and traces of lipids. The sediment was dried in the air and suspended in 20 ml. *N* potassium hydroxide. It was stirred at room temperature for 45 min. and then incubated for 18 hr. at 38° C. Insoluble fractions were spun down by high speed centrifugation. The clear yellow supernatant was used for acid fractionation and the chemical analyses as described above.

TABLE I

EFFECT OF PROLONGED ALDRIN FEEDING ON THE NUCLEIC ACID PHOSPHORUS OF LIVER TISSUE

Ser. no. of expts.	No. of mice	Days/p.p.m. aldrin feeding	Liver weight, mgm.	L/B*	H_2O in %	Mgm. P/1 gm. fresh tissue			Mgm. P/1 gm. dry tissue			PNA/DNA
						Total P	PNA	DNA	Total P	PNA	DNA	
Control (1-15)	15	0	1460 ± 55.45	0.058 ± .0016	65.3 ± 1.1	2.092 ± .056	1.417 ± .045	0.674 ± .030	6.113 ± .279	4.150 ± .208	1.965 ± .105	2.15 ± .120
Aldrin												
1	1	13/50	1592	.10	70.2	1.998	1.120	0.878	6.704	3.758	2.946	1.27
2	1	14/50	3319	.16	68.1	2.199	1.187	1.012	6.893	3.721	3.172	1.17
3	1	14/50	3728	.16	65.7	2 160	1.133	1.027	6.297	3.303	2.994	1.10
4	1	14/50	3239	.14	67.5	2.039	1.199	0.840	6.273	3.689	2.584	1.42
5	1	14/40	2860	.11	68.1	2.159	1.209	0.950	6.767	3.789	2.978	1.27
6	1	14/40	2358	.11	66.5	2.759	1.562	1.197	6.235	4.662	3.573	1.30
7	1	14/40	2366	.11	68.7	1.439	1.319	1.120	7.792	4.214	3.578	1.17
8	1	15/40	3063	.14	68.7	1.758	1.187	0.571	5.616	3.792	1.824	2.07
9	1	15/40	2807	.15	70.9	2.100	1.463	0.637	7.216	5.027	2.189	1.43
10	1	17/40	2761	.12	70.9	2.020	1.209	0.811	6.942	4.155	2.787	1.49
11	1	17/40	2952	.13	63.0	1.980	1.232	0.748	5.351	3.329	2.022	1.65
12	1	18/40	2858	.14	67.4	2.560	1.518	1.042	7.852	4.656	3.196	1.46
13	1	18/40	2545	.10	70.0	2.520	1.540	0.980	8.425	5.147	3.278	1.57
14	1	25/30	4683	.15	64.5	2.359	1.100	1.259	6.645	3.099	3.546	0.87
15	1	25/30	3760	.14	60.2	2.400	1.364	1.036	6.030	3.427	2.603	1.32
Total	15											
Mean				.13 ± .00654	67.4 ± .466	2.163 ± .0849	1.289 ± .0421	0.941 ± .050	6.736 ± .217	4.005 ± .125	2.885 ± .135	1.37 ± .070

Note: $\sqrt{(X-\bar{X})^2/(n-1)}$.

* *Liver/body ratio.*

Results

Table I contains the summarized results for the distribution of DNA and PNA in the liver tissue of mice kept on normal laboratory chow, and detailed results for the experimental mice kept on the same diet with the addition of aldrin.

Results are expressed in mgm. phosphorus per 1 gm. fresh and dry tissue and related to total nucleic acid phosphorus and DNA as well as PNA phosphorus. Values for inorganic phosphorus were quantitatively negligible and are not represented in the table. From Table I, it can be seen that feeding aldrin to mice, in different amounts and for various time intervals, induced an increase in the liver parenchyma accompanied by a highly significant increase in DNA phosphorus. On the other hand, the content of PNA phosphorus remained practically unaltered and this resulted in a drop in the PNA to DNA ratio. The slight elevation in the total nucleic acid phosphorus was derived almost entirely from the increase in DNA.

TABLE II

EFFECT OF PROLONGED ALDRIN FEEDING ON THE NUCLEIC ACID PHOSPHORUS OF BRAIN TISSUE

	No. of mice	Mean of brains in mgm.	Mgm. P per 1 gm. fresh tissue				
			Total P	Inorg. P	PNA P	DNA P	PNA/DNA
Control	12	454 ± 6.18	2.618 ± .0871	0.061 ± .0044	1.296 ± .0734	1.180 ± .0812	1.1 ± .124
Experiment 30 p.p.m. aldrin 14 days	10	486 ± 5.05	2.648 ± .146	0.069 ± .0071	1.318 ± .120	1.192 ± .067	1.1 ± .030

The results of estimations for nucleic acid phosphorus in the mouse brain are given in Table II. They include the summarized results of 12 normal and 10 experimental animals and are expressed per gram of fresh brain tissue. It is apparent that neither the amount of aldrin ingested nor the duration of feeding period has any bearing on DNA or PNA content of the brain tissue. All the results derived from experimental animals are comparable to those of the control animals. However, a slight, significant increase is noted in the weight of the brain of experimental animals.

Discussion

The information available at present concerning the estimation of nucleic acids in mouse tissues is mostly restricted to the results of Reddy and Cerecedo (12, 13). Their procedure, consisting of Schneider's trichloroacetic acid extraction (16) followed by a pentose estimation for PNA made according to the method of Euler and Hahn (7) and by a desoxypentose determination for DNA by Stumpf's method (17), differed from the procedure which was based on phosphorus determination. Nucleic acid estimations based on phosphorus

determination in general show higher value and this is probably responsible for the higher nucleic acid concentrations obtained in the present investigations.

Reddy and Cerecedo's recorded PNA and DNA concentrations per gm. normal dry liver tissue are 26.4 mgm. and 11.2 mgm. respectively. In this laboratory the concentrations of the corresponding nucleic acids per gm. normal dry liver tissue were estimated on the basis of phosphorus analyses, multiplying the values obtained by the factor 10.6 for PNA and 10.1 for DNA, according to Schmidt and Thannhauser. The amounts calculated in this way were 43.9 mgm. for PNA and 19.8 mgm. for DNA. These concentrations exceed those recorded by Reddy and Cerecedo; the ratio of PNA to DNA was however practically the same in the two investigations.

Previous workers have shown that concentrations for PNA and DNA are increased in malignant tissues of the mouse and also in the liver parenchyma of the tumor-bearing host (12, 13). The investigations of Annau and Manginelli (3) showed that tumor development in mice induced a progressive enlargement of the liver, a result of increased mitotic activity in that organ. In the mouse embryo, PNA and DNA concentrations are highest on the 12th day and then fall sharply. During gestation period there is also gradual increase in PNA as well as in DNA in the liver of the mouse (12, 13).

PNA and DNA estimations carried out in this laboratory showed that the prolonged feeding of aldrin to mice induced an increase only in DNA, while PNA remained unaltered although there was a considerable increase in the liver parenchyma. These results seem to indicate that there may be a biochemical difference between proliferative processes elicited by the presence of aldrin, and those related to physiological organ development, growth, or malignancy. This suggestion is corroborated further by previous observations which showed that feeding of aldrin induces a decrease in alkaline phosphatase and xanthine oxydase activities (1).

The aldrin feeding seemed to be without any effect on PNA and DNA content in the brain tissue of the mouse. An explanation for this may be found in the observation by Hevesy and Ottesen (10) and Samuel *et al.* (14), that nucleic acid metabolism is very slow in the nervous system and is determined by the rate of phosphorus turnover.

Before, however, any conclusion can be drawn, the validity of the method employed has to be established. Logan, Mannel, and Rossiter (11) showed recently that all PNA estimations carried out in nervous tissue according to the method of Schneider (16) and particularly by the method of Schmidt and Thannhauser (15) represent an overestimate. They stated that this discrepancy is due mostly to the diphosphoinisitide lipid–protein complex, present in large amount in the nervous tissue. According to Folch and Le Baron (9), it is the phosphorus of this compound which appears quantitatively in the ribonucleic acid phosphorus fraction under the conditions employed in the method of Schmidt and Thannhauser. The DNA phosphorus fraction, however, remains unaffected by this method and gives comparable results.

Considering these observations one has to conclude that PNA phosphorus concentrations, as determined in this laboratory, were also too high. Actually 1.29 and 1.18 mgm. phosphorus were estimated per gm. fresh brain tissue for PNA and DNA respectively, showing a ratio of 1.1 for PNA to DNA phosphorus. Consequently if we take for granted that PNA phosphorus concentrations were overestimated we have to suppose that also DNA phosphorus values obtained were too high, otherwise DNA phosphorus would exceed the amount of PNA phosphorus, which appears to be unlikely. Furthermore it may be assumed that PNA as well as DNA fractions were contaminated with "inositide phosphorus", though to different degrees. Although the occurrence of such a contamination cannot be excluded it seems to be improbable since according to the above mentioned authors only PNA fractions are affected by this component.

Summary

Prolonged feeding of aldrin to mice induced a proliferative increase in the liver parenchyma. Estimations of nucleic acid phosphorus in the liver of experimental and control mice indicated that aldrin feeding induced an increase in DNA phosphorus, while PNA phosphorus remained unaltered. The proliferative processes in the liver of mice produced by prolonged feeding of aldrin differed from similar processes elicited by malignancy or gestation.

Analyses for nucleic acid phosphorus in the brain tissue of aldrin fed mice did not reveal any change in DNA and PNA phosphorus. A light increase in the weight of the brain was noted in mice fed on aldrin.

Acknowledgments

I wish to thank Miss Grace Walker for technical assistance with the chemical aspects of this work and Dr. Chas. A. Mitchell, Chief of the Division, for his interest in connection with the problem.

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ADSORPTION OF ORANGE II BY CYTOCHROME *c* AND RIBONUCLEASE[1]

By J. Ross Colvin

Abstract

The adsorption isotherms of Orange II on cytochrome *c* and ribonuclease at pH 5.5 in 0.05 *M* acetate buffer are sigmoid. They may be interpreted by a previously described theory of interacting hydration effects. Adsorption of methyl orange or sodium flavianate by either protein was negligible.

Introduction

Within the last decade, adsorption of ions by proteins, particularly the serum albumins, has been investigated extensively (7). Where binding is detectable, the isotherms observed are usually good approximations to Langmuir hyperbolae and may be interpreted satisfactorily by assuming Coulomb forces between the ion and independent or interacting groups on the protein, supplemented by hydrogen bonding, ion-dipole, and dispersion forces. Recently, however, Karush (6) observed a sigmoid isotherm for *d*-phenyl-(*p*-aminobenzoylamino)-acetic acid on bovine serum albumin and Colvin (3,4) reported similar curves for the anions of methyl orange, Orange II, and flavianic acid (2,4-dinitro-1-naphthol-7-sulphonic acid) on such positive proteins as lysozyme, calf thymus histone sulphate, and protamine sulphate. Wetlaufer and Stahmann (10) have also observed sigmoid curves while studying the precipitation of positively charged polylysine by methyl orange and similar anions. As sigmoid isotherms must be a reflection of co-operative* behavior within the system, the possible adsorption of the anions of methyl orange, flavianic acid, and Orange II to the positive proteins cytochrome *c* and ribonuclease was of interest. The results of experiments made to estimate such adsorption are reported here.

Methods and Materials

All isotherms were estimated by the dialysis equilibrium technique which involves determination of the distribution of dye anions between a buffer solution on one side of a differentially permeable membrane and a protein solution on the other (8). Unless otherwise stated, sodium acetate – acetic acid buffer, pH 5.5, was used in all experiments and the concentration of buffer ions was sufficient (0.05 *M*) to reduce the Donnan effect to negligible proportions. Methods were as described previously (3) except that the total volume of liquid within the vials was 6 ml. Vials were rotated for six hours

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* *In this report, co-operative is used as in Fowler and Guggenheim's "Statistical Thermodynamics". It means that the interactions of the molecules or ions under discussion are so strong that the state of any one is fundamentally influenced by which states of the others are occupied.*

in a water bath at 32.0 ± 0.1° C. Protein concentration was 0.2% for cytochrome *c* and 0.1% for ribonuclease. Molecular weight of both proteins was assumed to be 13,000 (2,11). Because of shortages of material, the average numbers of anions bound per protein molecule are uncorrected for moisture in the sample of protein and are therefore minimum estimates. This, however, does not affect the validity of the interpretation of the results.

The cytochrome *c* was an amorphous product purchased from General Biochemicals, Inc. and used without further purification. The isoelectric point of cytochrome *c* was assumed to be 10 (11). The pH of a 0.2% solution of the protein in distilled water was 8.0. At pH 6.8 in 0.05 *M* phosphate buffer the samples of cytochrome *c* gave a single peak in the ultracentrifuge and in the electrophoresis apparatus.

Ribonuclease was a crystallized product, prepared from bovine pancreas by Armour and Co., Chicago, and used without further purification. It too gave a single peak in the ultracentrifuge in 0.05 *M* phosphate buffer at pH 6.8 and in the electrophoresis apparatus at pH 5.5 in 0.05 *M* acetate buffer. The isoelectric point reported for ribonuclease at the concentrations of salt used here is about pH 7.8 (9). The pH of a 0.2% solution of the protein in distilled water was 5.6. Precipitation of the positive protein-anion complex, which always occurred when adsorption was detectable, did not change the pH of the solutions appreciably and the complexes redissolved readily upon sufficient dilution of the buffer.

Sodium salts of the anions were prepared and purified as previously described (3).

All buffers were prepared by Clark's method (1) from reagent grade materials and checked against commercial standards.

Results

Adsorption of Orange II by cytochrome *c* at pH 6.8 in 0.05 *M* phosphate buffer was slight (Fig. 1). Decreasing the pH to 5.5 in 0.05 *M* acetate buffer, however, greatly increased the amount of Orange II bound and converted the lower portion of the isotherm to an approximately exponential form (Fig. 1). In contrast, adsorption of methyl orange by cytochrome *c* at pH 5.5 and 6.8 in 0.05 *M* acetate and 0.05 *M* phosphate buffers respectively was negligible. Adsorption of the anion of flavianic acid to cytochrome *c* at pH 5.5 could not be detected up to free anion concentrations of 13×10^{-4} *M*.

Adsorption of Orange II by ribonuclease at pH 5.5 was appreciable and described by a sigmoid isotherm (Fig. 2). Intensity of adsorption and total binding capacity of ribonuclease for Orange II was less than that of the more basic cytochrome *c* for the same anion although the two proteins are equal in size (Compare Figs. 1 and 2). Methyl orange was bound negligibly to ribonuclease in 0.05 *M* acetate buffer, pH 5.5, and at pH 6.8 in 0.05 *M* phosphate. No evidence was found for adsorption of the anion of flavianic acid to ribonuclease.

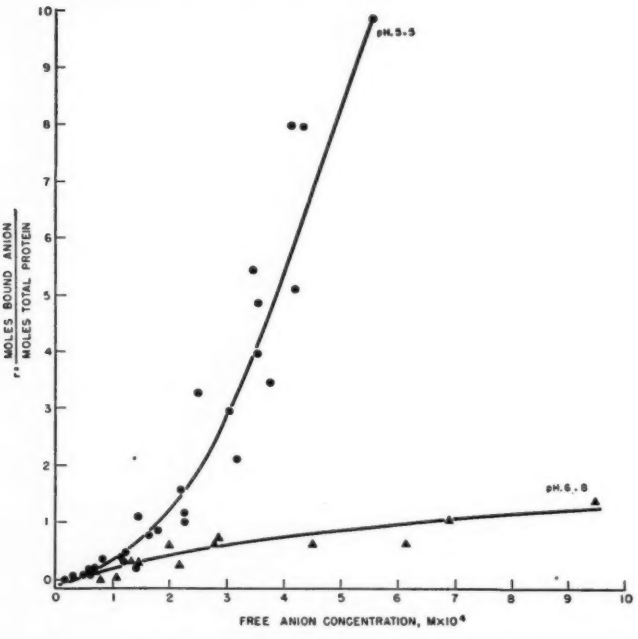

FIG. 1. Adsorption isotherms at 32° C. of Orange II on cytochrome *c* in 0.05 *M* phosphate buffer, pH 6.8, and in 0.05 *M* acetate buffer, pH 5.5.

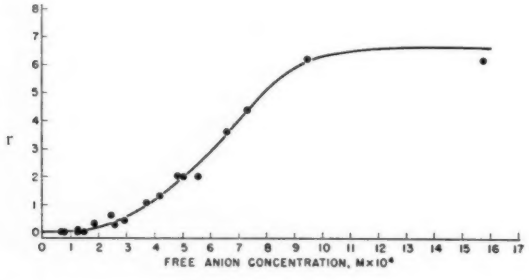

FIG. 2. Adsorption isotherm of Orange II on ribonuclease in 0.05 *M* acetate, pH 5.5, 32° C.

Repeated attempts to determine the adsorption isotherms for all systems at low ionic strengths (0.001) gave erratic results because of the low buffer capacity of the solvent superimposed upon the Donnan effect. However, appreciable adsorption of Orange II to both cytochrome *c* and ribonuclease was apparent qualitatively in 0.001 *M* acetate, original pH 5.5.

Discussion

These results extend previous observations and conclusions on similar systems. Under the conditions described, the smaller anions of methyl orange and flavianic acid are not adsorbed perceptibly by either cytochrome *c* or ribonuclease, although flavianic acid is a very common precipitant for basic compounds. The larger anion of Orange II is appreciably adsorbed to both proteins at lower pH values (5.5) but adsorption is greater on the more basic cytochrome *c*. Thus, as might be expected from prior studies (3,10), increasing anion size, increasing basicity of the protein, and decreasing pH promote adsorption of an anion to positive proteins accompanied by precipitation of the complex. In addition, whenever adsorption is appreciable, the isotherm is sigmoid. Together with previous studies (3,4,9), the present results suggest that sigmoid isotherms are a characteristic property of anion-positive protein systems, in contrast to the Langmuir hyperbolae which are found with anion-negative protein interactions (7,8).

The sigmoid isotherms have received two different interpretations. In the first, a formal thermodynamic description of such functions has been given by assuming an ideal solubility product relation, $P^{n+} + nA^- \rightleftarrows PA_n$ (PA_n insoluble), provided that the initial portion of the isotherm is attributed to polydispersity of the adsorbing material. On this basis precipitation curves may be constructed which fit the data reasonably well after assuming a proper choice of constants. This approach has been applied by Wetlaufer and Stahmann (10) to the system methyl orange – polylysine. In the second interpretation, interacting hydration effects, whereby the adsorption of one anion to a small positive protein molecule promotes the adsorption of another, have been assumed. Co-operative interactions of this type permit the qualitative prediction of sigmoid isotherms and the binding of several anions by a number of basic proteins has been resolved in this way (3,4). The same exposition is valid without change for the present systems.

In the present study, the second interpretation is preferable for the following reasons. Although the possibility of heterogeneity of the adsorbent can never be completely excluded, there appears to be no justification for assuming marked heterogeneity in either sample of the proteins studied. Furthermore, the solubility product method of description offers no explanation for the absence of intermediate complexes PAx, $(x < n)$, which would be expected both from electrostatics and from previous work with the same anions on negative proteins (6,7,8). Finally, as recently shown by Hill (5) in an approximate theoretical treatment, the rejection of the exponential portion of the sigmoid isotherm may be unwarranted. Provided that attractive interactions between adsorbed molecules or equivalent effects are present, sigmoid isotherms are to be expected, accompanied by a first order phase change. These considerations support the assumption of co-operative interactions between components of the system, but of course do not exclude application of the solubility product principle. Additional experimental evidence is needed to distinguish between the two possibilities.

Acknowledgments

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THE DETERMINATION OF SERUM PROTEIN FRACTIONS ON FILTER PAPER ELECTROPHEROGRAMS BY THE BIURET REACTION, AND SOME OBSERVATIONS ON THE SERUM PROTEINS OF THE ESTROGENIZED IMMATURE PULLET[1]

By W. P. McKinley[2], W. A. Maw[3], W. F. Oliver[4], and R. H. Common[5]

Abstract

An application of the biuret reaction to the determination of protein fractions on filter paper electropherograms of serum is described. The relative mobilities of the serum protein fractions of the domestic fowl and of man are compared. Values are reported for serum protein fractions as separated by filter paper electrophoresis in a methanolic veronal buffer. Some observations on the serum proteins of the estrogenized immature pullet are reported; and it is tentatively suggested that another fraction as well as serum phosphoprotein appears in the serum of the pullet as a consequence of treatment with estrogen.

Introduction

Cremer and Tiselius (4) determined protein fractions on filter paper electropherograms by staining with dyestuff, subsequently eluting dyestuff bound by the protein and determining the dyestuff photometrically. The dye-binding capacity of globulin was less than that of albumin in the sera studied. Cremer and Tiselius used correction factors to bring the optica' densities corresponding to albumin and globulin to the same protein basis. Flynn and DeMayo (5) have stressed the necessity for careful standardization of the time of staining if the dyestuff is to be eluted for quantitative purposes. In our experience both the rapidity and temperature of drying the papers also affect the subsequent staining and should, therefore, be kept as constant as possible.

The validity of correction factors determined for normal sera may be questionable if highly lipemic sera are being studied (3). This possibility is equally cogent if protein is determined by scanning the stained papers directly with a densitometer. Some workers (9) have preferred to cut up the papers in appropriate portions and to determine nitrogen by a Kjeldahl method. Ganzin and Macheboeuf (6) have determined the protein fractions by eluting them from the papers with saline buffered by sodium carbonate to pH 8.65 and then determining protein by the phenol reagent of Folin and Ciocalteu. However, the Kjeldahl method is laborious when it is necessary to dissect papers into numerous strips, while the reagent of Folin and Ciocalteu is rather unspecific. The present paper describes an application of the quantitative biuret reaction to the determination of protein fractions on filter paper electropherograms and presents the results of experiments with avian sera and egg yolk.

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Experimental Methods

Technique of Electrophoresis

The apparatus was constructed from glass, stainless steel angle rod, and "Perspex"; it was similar to that described by Flynn and DeMayo (5), except that the papers were supported in the horizontal position. Veronal buffer (pH 8.6; ionic strength $\mu = 0.05$) was used as the standard working buffer. In addition many experiments were made using a veronal buffer made up in the same proportions except that 20% methanol was used instead of water. This methanolic buffer has been found advantageous for the separation of phosphoprotein fractions from the sera of estrogenized or laying birds (3, 10).

All the electrophoretic experiments described in the present paper were conducted in a cold room maintained at 6° to 7° C. Under these conditions a potential of 200 volts with a current of 9 to 10 ma. has given satisfactory results with Whatman 3MM filter papers 8.5 in. wide and 16 in. long. The papers were run as a rule for 24 hr. or longer. Ten hours was sufficient to provide resolution into albumin, α_2 globulin, and β and γ globulins, but 24 hr. was necessary to secure adequate distinction of the α_1 globulin and α_3 globulin fractions, and to reveal further resolutions described below.

The technique of application of the protein solution to the papers was found to be critical. The buffer was allowed to rise in the paper from the anodic compartment to within about 1 cm. of the line of application; then the other end of the paper was allowed to dip into the cathodic compartment. When the two buffer fronts were about $\frac{1}{2}$ in. apart, the sample was applied from a micropipette along the narrow, unwetted zone. This was always done so that only a few minutes were needed for the buffer fronts to coalesce with the sample. The current was then applied. In many experiments as much as 0.5 ml. serum was taken on wide strips in order to work on a sufficiently large scale, and to have a number of comparable strips for staining by different methods.

On completion of the run, the paper was brought out of the apparatus and a strip 1 in. wide cut off either side. The remainder was at once replaced in the electrophoresis chamber, but without letting the ends of the paper dip into the electrode compartments. The strips were dried and rapidly stained in a saturated solution of naphthalene black in methanol containing 10% acetic acid. Since these strips were stained merely to locate the principal fractions, the operation could be completed in about 40–45 min. The pattern revealed by staining then served as a guide for the dissection of the unstained, undried part of the paper into strips carrying one only, or mainly one only, protein fraction.

Determination of Proteins

The protein was eluted from these strips as follows:—

Ten milliliters of 1% saline was taken in each of a series of small vials. The strips from the electropherogram were folded and the strip thus folded

was in turn enfolded in a strip of filter paper about 1.5 in. wide. This strip served as a wick, and the two pieces of filter paper were held together by a paper clip. The free end of the wick was then placed in the vial of saline, while the end enfolding the piece of the electropherogram was allowed to hang into a receiving test tube marked at 5 ml. A period of two hours was sufficient to complete the elution. Experiments expressly made showed that at least 96% of the total serum protein could be recovered from the paper, while staining tests failed to detect any protein on the residual paper. Ganzin and Macheboeuf (6) have demonstrated the possibility of eluting protein fractions from electropherograms with small volumes of saline adjusted to pH 8.65 with carbonate buffer, and such buffered saline is probably preferable to the unbuffered saline used by us.

The volumes of the eluates were checked and made up to 3 ml. if necessary. The biuret color reaction was developed by the procedure of Weichselbaum (15) as follows:—To each tube was added 3 ml. Weichselbaum's quantitative biuret reagent. The contents were mixed and placed for *exactly* 30 min. in a water bath maintained at 32° ± 0.5° C. The tubes were then removed and quickly cooled in ice water. The optical densities were read in the Evelyn colorimeter using the "520 M" filter. The blank was prepared by eluting a portion of the filter paper treated in the same way as the parts bearing the protein. It was found convenient to use a piece of the paper cut from the cathodic end. The blanks gave a small but consistent absorbency when read against 3 ml. saline treated with 3 ml. biuret reagent.

Standard curves of optical density against protein (N × 6.25) were prepared by the procedure of Wolfson *et al.* (16) using pooled sera from eight immature pullets and pooled sera from five heavily estrogenized immature pullets (2 mgm. estradiol benzoate intramuscularly on each of four successive days). The desired portion of pooled serum was made up with 1.0% saline to a volume of 5 ml. and 5 ml. of the biuret reagent was added. After color development the optical density was read in the Evelyn colorimeter using a "520 M" filter. The standard curves obtained for the two composite samples of blood did not differ (*vide* Fig. 1). Veronal does not interfere with the biuret reaction. Ethanolamine does interfere, and ethanolamine buffers cannot be used in conjunction with the biuret reaction, although they permit of excellent separation on paper electropherograms.

The optical densities corresponding to the separate fractions were expressed as percentages of their sum. The total serum protein was determined on a separate sample of serum, and the percentage partition was then translated into terms of gm. protein per 100 ml.

The quantitative validity of the results is contingent on the validity of the assumption that the relation between biuret color and protein nitrogen was substantially the same for all fractions studied. The phosphoprotein fractions suggested themselves as the most likely to introduce error in this way. Accordingly, a crude lecithovitellin solution was prepared by diluting serum of

estrogenized pullets with water (1 : 1), separating the precipitate on the centrifuge and resuspending the precipitate in 1.0% sodium chloride. The plot of optical density against protein nitrogen for this preparation was practically the same as for the total protein of serum (*vide* Fig. 1). With one or two very highly lipemic sera a slight divergence was noted; this was probably due to turbidity resulting from the lipid content of the solution. Treatment of the solution with ether was not effective for removal of this turbidity. However, the results showed that the error introduced by using the curve for total serum protein for determining the amount of phosphoprotein was negligibly small in the present experiments.

The scale of working as described above was limited by the use of the Evelyn colorimeter. A considerable reduction could probably be attained by basing the analysis on measurement of the absorption in the near ultraviolet as described by Goa (7). It is advisable to check standard curves for each new batch of reagents.

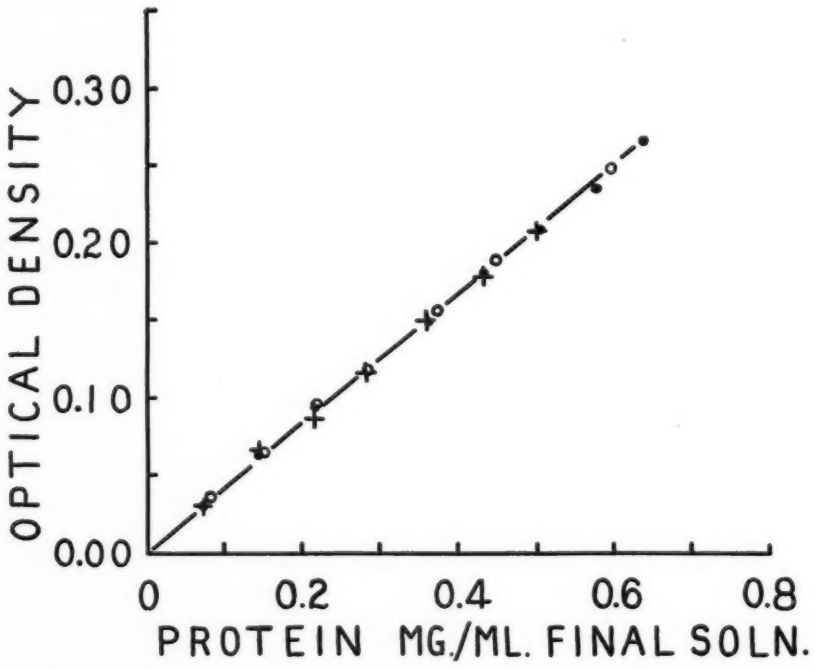

FIG. 1. Typical calibration curve of biuret color against protein (N × 6.25) concentration in final reaction mixture.

● Control serum
○ Serum from estrogenized pullets
× Lipophosphoprotein precipitate from diluted serum

Experimental Results

A Comparison of the Relative Mobilities of Avian Serum Protein Fractions in Aqueous and Methanolic Buffers

The movement of proteins on filter paper electropherograms is determined not only by the factors of charge, density, viscosity, and current which operate in classical electrophoresis, but also by electroendosmosis and solvent evaporation. Adsorption may also play a part, but any such effects are believed to be slight. Holt *et al.* (8) have demonstrated the influence of the time of running the experiment on the apparent relative mobilities of proteins in filter paper electrophoresis. More recently the electroendosmotic and evaporative effects have been studied by Macheboeuf *et al.* (11) and utilized to secure movements of proteins independent of the point of application. However, careful control of current, stabilization of temperature conditions, and close adherence to standard manipulative technique and time of running permitted of a remarkable reproducibility of relative mobilities in the present experiments.

The relative (12) mobility of a phosphoprotein fraction present in the sera of laying or estrogenized birds was known to be profoundly modified by substitution of methanolic for aqueous buffer (3, 10). It became important, therefore, to study the effects of the methanolic buffer on the relative mobilities of the other fractions. Comparisons of average values for pullet and for human sera using either buffer are presented in Table I.

TABLE I

RELATIVE MOBILITIES OF PROTEIN FRACTIONS ON FILTER PAPER ELECTROPHEROGRAMS USING AQUEOUS VERONAL BUFFER, pH—8.6, IONIC STRENGTH 0.05, OR A VERONAL BUFFER MADE UP SIMILARLY BUT WITH 20% METHANOL AS SOLVENT INSTEAD OF WATER. PAPERS RUN FOR APPROXIMATELY 30 HR. BIRDS AGED 7 TO 15 WEEKS

Protein fraction	Fowl sera				Human sera	
	Control pullets		Estrogenized pullets**		Aqueous buffer (5)	Methanolic buffer (6)
	Aqueous buffer* (1)	Methanolic buffer (2)	Aqueous buffer (3)	Methanolic buffer (4)		
No. of samples	14	14	8	14	4	4
Albumin	1.00	1.00	1.00	1.00	1.00	1.00
α_1 globulin	0.73	0.84	0.8–0.9	0.85	0.87	0.84
α_2 globulin	0.59	0.64	0.64	0.64	0.70	0.62
α_3 globulin	0.48	0.51	0.50	0.50	Not observed	Not observed
β globulin	0.36	0.38	0.38	0.38	0.47	0.46
γ globulin	0.27	0.27	?	0.26	0.17	0.17
PP_1	—	—	?	0.17	Absent	Absent
PP_2	—	—	?	0.11	Absent	Absent

* *The results for control pullets in aqueous buffer have been reported previously (3).*

** *The results for estrogenized pullets were obtained on birds that had received 1 mgm. estradiol benzoate (Progynon B, Schering) intramuscularly per day for three successive days.*

A comparison of the values in columns 1 and 2 showed that the average relative mobilities obtained with methanolic buffer did not differ greatly from those obtained with aqueous buffer. A comparison of columns 5 and 6 showed that this was true also for human sera. A comparison of columns 3 and 4 showed that approximately the same relative mobilities were obtained for the α globulins and β globulin in both buffers. However, in aqueous buffer the γ globulin region was obscured by lipoprotein ("PLV" fraction) to the extent that neither γ globulin nor PLV fraction could be distinguished with any certainty. In contrast with this, the methanolic buffer enabled the position of the globulin to be distinguished, and its relative mobility was then similar to its mobility in the control sera. At the same time the double presumptive phosphoprotein ("PP") fraction was clearly defined. Thus the methanolic buffer had permitted the segregation of this double "PP" fraction without materially affecting the relative mobilities of the other major protein fractions.

Quantitative Partition of Serum Protein in Fowl Sera

The partitions of proteins in sera from control and estrogenized immature pullets were determined as described above. Average values are presented in Table II.

TABLE II

AVERAGE VALUES FOR SERUM PROTEINS OF PULLETS AGED 7 TO 12 WEEKS. PROTEINS FRACTIONATED BY FILTER PAPER ELECTROPHORESIS. VERONAL BUFFER, pH 8.6, MADE UP WITH 20% METHANOL

	Controls		Estrogenized*	
	Average for 13 birds		Average for 13 birds	
	% of total	Gm./100 ml.	% of total	Gm./100 ml.
Albumin	44.0	1.61	36.0	1.46
α_1 globulin	2.0	0.07	2.4	0.09
α_2 globulin	9.3	0.34	9.4	0.38
α_3 globulin	6.1	0.22	3.7	0.15
β globulin	17.6	0.64	10.2	0.41
γ globulin	21.0	0.77	19.8	0.81
"PP" fractions	Nil	Nil	18.5	0.75
Total	100.0	3.65	100.0	4.05
Ratio $\frac{\text{albumin}}{\text{total other protein}}$	0.79	—	0.56	—
Ratio $\frac{\text{albumin}}{\alpha + \beta + \gamma \text{ globulins}}$	0.79	—	0.79	—

* *1.0 Mgm. estradiol benzoate (Progynon B, Schering) per day for three days.*

The increase of average total serum proteins brought about by estrogen was subject to the disturbing effect of lipemia, which of itself would tend to depress serum protein. This lipemia may attain extraordinarily high levels, as is shown by results for the total alcohol–ether soluble material of avian sera recorded elsewhere (2), but quoted here in Table III for convenience.

TABLE III

EFFECTS OF GONADAL HORMONES ON TOTAL SERUM LIPIDS OF SEXUALLY IMMATURE PULLETS (2). HORMONES ADMINISTERED ON ALTERNATE DAYS OVER AN EXPERIMENTAL PERIOD OF 12 DAYS. BIRDS AGED 13 TO 15 WEEKS

No. of birds	8	8	8	8
Total dosage of: ODB, mgm.	Nil	Nil	6 × 1.0	6 × 1.0
TST*, mgm.	Nil	6 × 1.0	Nil	6 × 1.0
Total serum lipid, gm./100 ml.	0.27	0.27	13.1	11.9**

* *Testosterone propionate (Oretone, Schering).*

** *Serum lipid with both hormones was significantly ($P < 0.05$) less than with estrogen only.*

It is evident from Table II that a moderate treatment with estrogen (1 mgm. ODB on each of three successive days) reduced the crude A/G ratio from 0.79 to 0.56. An approximate correction for phosphoprotein may be applied by omitting the PP fraction in calculating the A/G ratio, and when this was done the average "corrected" A/G ratio was approximately the same as for the control birds. Clearly part at least of the decrease in A/G ratio can be ascribed to the appearance of the "PP fraction". The fact that the numerical values for the control and "corrected" A/G ratios are the same might suggest that the PP fraction accounts for the decrease entirely. However, it should be remembered that the γ globulin zone is normally wider than any other protein zone on the electropherograms. Hence the PP zone on dissected papers may include part of the trailing γ globulin zone, thus exaggerating the increase of A/G ratio on correcting for phosphoprotein. In addition, the results in Table II relate to relatively moderate estrogen treatments. The data in Table II do not, therefore, exclude the possibility that there is also some increase in the γ globulin zone. It will be shown below that there is evidence for such an increase in the sera of more strongly estrogenized pullets. The main point to be made here is that a considerable proportion at least of the decrease in A/G ratio is due to the appearance of a new PP fraction, a fraction which is separable to a large extent by running the electropherograms with methanolic buffer.

The "PP" fraction may be separated from the serum of estrogenized pullets by diluting the serum with one volume of water and then centrifuging. Fig. 2 presents electropherograms obtained with the use of methanolic buffer and stained with naphthalene black. The patterns given by the sera of estrogenized pullets (Fig. 2 *a*), and laying hen (Fig. 2 *b*) were strikingly similar, and displayed the double nature of the PP zone. The electropherogram of the suspension of the precipitate in 1.0% saline displayed a double band in a position closely approximating that of the double PP band of the serum electropherograms.

The Number of Fractions Distinguishable on Electropherograms of Fowl Sera

The results in Table I and Table II were based on visual location of the protein fractions. Six major fractions were formed in electropherograms of sera of four control pullets when the experiments were run in methanolic or aqueous buffer for approximately 30 hr. under the conditions specified. Acquisition of a Photovolt Densitometer (Model 525) permitted more detailed studies. Color densities on Whatman 3 MM papers stained with naphthalene black may be measured with this instrument.

A typical densitometric plot for an electropherogram of a pullet aged three weeks is shown in Fig. 3. The albumin and the other five major fractions were distinct, and their relative mobilities were in reasonable agreement with the average values given in Table I and obtained under similar conditions. However, a number of subsidiary peaks were also visible on the plot. These might seem insignificant, but certain features were observed rather consistently. These features were as follows: (*a*) Two or three minor peaks following on the main α_1 position; two of these are indicated by arrows on

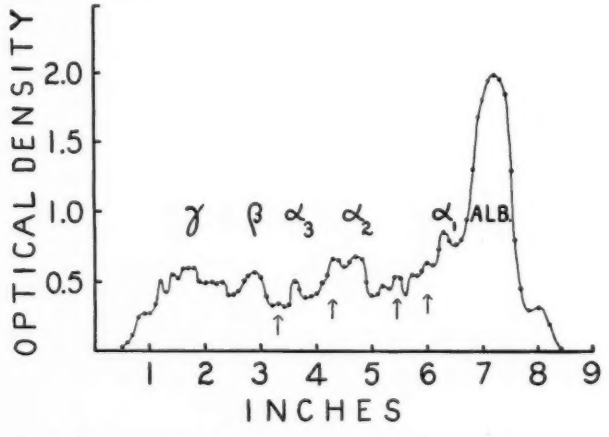

FIG. 3. Plot of optical density of electropherograms of serum of an immature pullet. Direct densitometry of paper stained with naphthalene black.

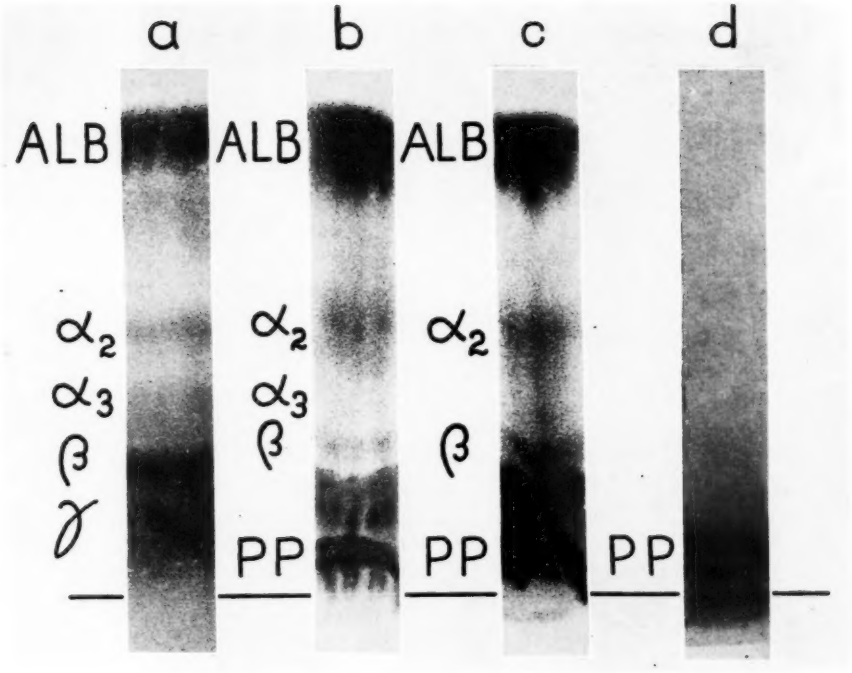

FIG. 2. Electropherograms (methanolic veronal buffer) of sera of domestic fowl. Stained with naphthalene black.

a. Immature pullet, control.
b. Immature pullet, estrogenized.
c. Laying hen.
d. Dispersion in saline of the precipitate obtained by diluting serum of estrogenized pullet with an equal volume of distilled water and centrifuging.

Fig. 3; (*b*) there were frequently two peaks in the α_2 region, the second being denoted by an arrow in Fig. 3; (*c*) the γ region displayed a tendency to split into a number of fractions.

Fig. 4 presents a plot of radioactivity against position for an electropherogram of a serum sample drawn from an immature pullet 12 hr. after feeding 2 mc. S^{35} labelled L-methionine in order to label the proteins by the method of Niklas and Maurer (14). The electropherogram was developed under the same conditions as were those to which Fig. 3 and the data in Tables I and II relate. It will be noted that there are definite peaks corresponding to the six major protein fractions; their positions are denoted by the appropriate letters. The relative mobilities correspond closely to the averages given for similar conditions n Table I and are as follows:

$$\alpha_1 = 0.84; \alpha_2 = 0.63; \alpha_3 = 0.53; \beta = 0.37; 0.19.$$

It will be noted that there are two minor peaks between the α_1 peak and the α_2 peak; one minor peak between α_2 and α_3 peaks; one minor peak between α_3 and β; and that there is a second β peak. These minor peaks are marked with arrows on Fig. 4. With the exception of the second β peak, these minor peaks correspond with those observed on the stained electropherograms (compare Fig. 3 and Fig. 4). Caution is necessary when interpreting such minor peaks, but the consistency with which they occurred appears to call for comment. No explanation is offered for the peak of radioactivity at the point of application in Fig. 4.

Densitometric measurements on papers stained with naphthalene black have revealed a small but consistent subsidiary peak preceding the albumin peak (*vide* Figs. 3 and 5). This peak does not appear to be the same as the "component 1" of sera of estrogenized birds described by Moore (13) and by Clegg and Hein (1) as present in the sera of laying or estrogenized birds,

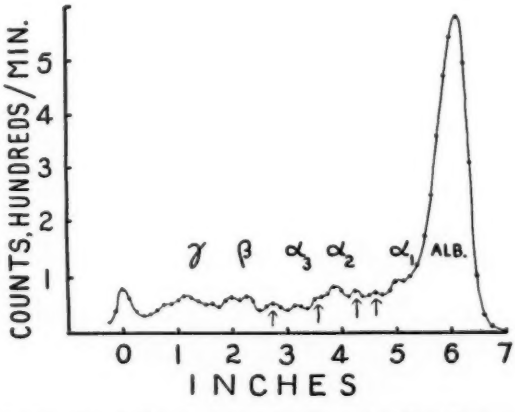

FIG. 4. Plot of radioactivity of electropherogram (methanolic veronal buffer) of serum of control pullet which had been given S^{35} L-methionine in order to label the proteins.

for it was detected about equally in sera of control and of estrogenized or laying birds; furthermore, labelling experiments (10) did not suggest that it was specially rich in phosphorus, whereas component 1 of Clegg and Hein (1) was strongly radioactive after injection of P^{32} labelled phosphate.

It has already been shown that the lipophosphoprotein ("PP") fraction may be readily separated on filter paper electropherograms of laying hens or estrogenized immature pullets by using a methanolic buffer. It has also been shown that a similar fraction, accounting for approximately one-third of the total proteins, may be separated from saline solutions of hen's egg yolk (10). These circumstances prompted an examination of the sera of estrogenized birds for a protein fraction corresponding to the constituent of the other two-thirds of the yolk protein. This search was also influenced by the hypothesis that the yolk proteins are synthesized in the liver and transferred to the ovary in the blood serum.

Fig. 5 presents densitometric plots of electropherograms of serum from (*a*) an immature pullet aged eight weeks and from (*b*) a similar pullet which received 2 mgm. estradiol benzoate on each of three successive days before

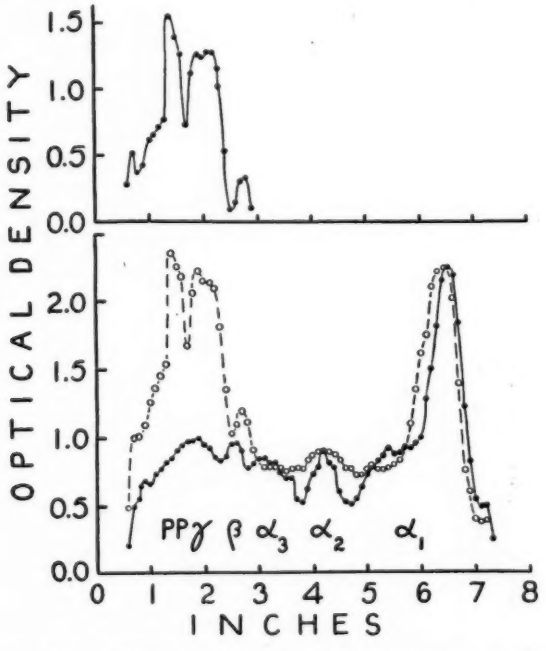

FIG. 5. Comparison of densitometric plots of electropherograms (methanolic buffer) of sera of control pullet (full line and dots) and of estrogenized pullet (broken line and circles). Curves adjusted to same amounts (approximately) of albumin. The upper curve is a plot of the difference between the foregoing two curves. Note peak suggesting presence of extra component in the γ zone as well as the sharp peak corresponding to the PP components.

blood sampling. For purposes of comparison both plots have been adjusted so as to correspond approximately to equal amounts of serum albumin, for the amount of serum albumin has been shown not to be appreciably affected by estrogen. The electropherograms were run under precisely similar conditions, and the correspondence of the major peaks as far back as the β globulin was remarkably close. The increase of the proteins in the γ globulin region was obvious, and the most marked feature was the appearance of the "PP" fraction. The double nature of the "PP" fraction could be seen on the paper but is not revealed by the plot, this failure being due to a certain amount of "tailing" of the trailing edges of the two subfractions of the "PP" fraction. On plotting the differences between the two curves, the curve thus derived could, with some plausibility, be divided between the "PP" fraction and a second fraction. This second fraction was of the order of twice the amount of the "PP" fraction. It is of interest to compare this result with the results for egg yolk. While it is, of course, possible that this second new moiety of the increase of protein due to estrogen may merely represent an increase of γ globulin, we are inclined to believe that it corresponds to a non-phosphoprotein yolk protein fraction. The relationship of one or other of the "PP" fractions to the phosphovitin of Mecham and Olcott (12) and the possible relationship of the second moiety to "livetin" are matters calling for further study. The present experiments, however, demonstrate that the increase of total globulin in the estrogenized bird and, by inference, in the laying bird is not solely due to the appearance of serum phosphoprotein, i.e. of the PP fraction. Both moieties of the increase are associated with phospholipid, as may be seen on referring to results secured using P^{32} and reported elsewhere (10).

Acknowledgments

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THE FAILURE OF GROWTH HORMONE TO MODIFY HORMONAL HYPERTENSION IN THE RAT[1]

By Sydney M. Friedman, Miyoshi Nakashima, and Constance L. Friedman

Abstract

Growth hormone (Connaught) was given in 1 mgm. daily doses to rats either otherwise untreated or given an amount of DCA or Compound F sufficient to elicit a moderate hypertensive response. At the dose level used, growth hormone did not affect either blood pressure or kidney size in treated or untreated animals. The dose used was one-fifth the amount found to increase renal function in the dog and 10 times the dose required for trophic effects in the rat.

Introduction

For some time now the hypophysis has been of interest in studies of hormonal influences in hypertension. A considerable body of information has accumulated concerning the vasopressor fraction of the posterior lobe, the most recent findings indicating the interesting, although paradoxical, fact that pitressin opposes both DCA (desozycorticosterone acetate) and Compound F (17-OH-corticosterone acetate) hypertensions (3, 4). Similarly, the anterior lobe has been under continuous scrutiny, especially since Selye reported that dried anterior pituitary powder (L.A.P.) had hypertensive and nephrosclerotic properties (6). These effects apparently do not reside in the A.C.T.H. fraction of the anterior pituitary, but rather, according to this author, are effects of the somatotrophic principle (7).

Despite these findings there has been surprisingly little animal experimentation concerning the cardiovascular–rental effects of growth hormone. Several years ago, White and his co-workers (9) pointed out that in the dog, growth hormone tended to increase renal function, although certain preparations were not so active as others (10).

It seemed to us of some importance to reassess the effects on the blood pressure and kidney of somatotrophic hormone given alone or in combination with other known hypertension producing hormones. Since our interest was in a possible physiological effect of this material in the animal body, the selection of dose seemed important. The two pertinent studies suggesting an appropriate dose were those of White *et al.* (10) in the dog and Beznák in the rat (2). In the dog, approximately 5 mgm. per day (0.5 mgm. per kgm.) proved a powerful stimulant of renal function. In the rat, Beznák obtained data on cardiac hypertrophy and hypertension with 100 μgm. per day. These two doses seemed in line with one another. In contrast, Selye (7) used 5 mgm. per day in the rat to obtain nephrosclerosis and hypertension. It seemed to us that such a dose was large enough to suggest that in this work

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the contaminants of the growth hormone preparation used might themselves have had considerable effect. If growth hormone itself had any significant nephrosclerosing and hypertension producing effect it would seem that it should be evident with a smaller dose. Accordingly, the work to be reported here was carried out using 1 mgm. per day in the rat. This is 10 times the dose used by Beznák.

As far as the growth promoting activity of this preparation is concerned, 25 μgm. per day is reported to increase the width of the proximal epiphyseal cartilage of the tibia in the 28-day-old hypophysectomized rat approximately 100 μ within four days on the average (1). This width is ordinarily about 200 μ in untreated animals. Further, a positive response was obtained with this test with as little as 4 and 10 μgm. per day administered for four days.

Experimental

Methods

Young male albino rats of an inbred Wistar strain were used throughout these experiments. They were maintained on Purina Fox Chow (Ralston). Growth hormone was supplied to us through the National Research Council of Canada as prepared by the Connaught Medical Research Laboratories. The preparation shows only the following minimal impurities:

T.H.	– less than 0.05 U.S.P. units per mgm.
A.C.T.H.	– less than 0.005 units per mgm.
Prolactin	– less than 0.2 I.U. per mgm.
Oxytocic activity	– 0.0007 units per mgm.
Pressor activity	– less than 0.02 units per mgm.

The preparation is accordingly very much purer than that used by any of the workers cited in the introduction.

In all cases where this growth hormone was administered, the daily dose was diluted in saline immediately prior to injection and 1 mgm. in 0.5 cc. of saline was injected subcutaneously.

DCA (desoxycorticosterone acetate) was administered by the subcutaneous implantation of pellets (one-quarter of a 75 mgm. Cortate pellet), three such pellets being implanted in the first week and three in the second.

Compound F (17-OH-corticosterone-21-acetate) was administered as a saline suspension, 2 mgm. per day being injected subcutaneously. This dose had previously been shown sufficient to elicit a hypertensive response in such young growing rats as were used here (5).

Blood pressure was determined by indirect tail plethysmography every second day beginning in the third week. This method was used only to establish group blood pressure averages in order to obtain guidance for terminating the experiment. At termination, blood pressure was determined by direct aortic cannulation using a 22 gauge needle coupled to a Sanborn electromanometer. Only the final direct blood pressure values are presented in the tables which follow.

Immediately at the conclusion of the direct pressure determination blood was taken from a side-arm connection to the aortic needle which was already in place and centrifuged immediately for subsequent sodium and potassium analysis. These cations were determined by flame photometry using a Beckman model B photometer.

Observations

Experiment No. 1

Thirty-two male albino rats weighing an average of 110 gm. were divided into four matched groups of eight. All animals in this experiment were uninephrectomized one week prior to beginning treatment. Group 1 served as untreated uninephrectomized controls, and group 2 received DCA by the subcutaneous implantation of pellets as described. Group 3 received 1 mgm. of growth hormone daily subcutaneously, and group 4 received both DCA and growth hormone. The experiment was terminated on the 31st day. Table I presents the pertinent data.

Growth hormone in the dose used here and in the size of rats chosen for this experiment did not increase body weight during the period studied.

TABLE I

	Group			
	Control	DCA	Growth hormone	DCA + growth hormone
Initial body wt., gm.	120 ± 12	107 ± 9	116 ± 11	98 ± 9
Final body wt., gm.	213 ± 14	198 ± 14	210 ± 5	190 ± 11
Direct aortic blood pressure, mm.Hg				
Systolic	125 ± 2	157 ± 11 $p < 0.01$	116 ± 2	151 ± 7
Diastolic	75 ± 2	95 ± 9 $p < 0.05$	69 ± 2	89 ± 6
Mean	99 ± 2	124 ± 10 $p < 0.01$	92 ± 2	120 ± 3
Kidney wt., mgm./100 cm.^{2}	316 ± 11	352 ± 10	281 ± 8	329 ± 16
Heart wt., mgm./100 cm.^{2}	203 ± 7	220 ± 9	200 ± 7	204 ± 9
Adrenal wt., mgm./100 cm.^{2}	8.7 ± 0.11	7.2 ± 0.06	9.3 ± 0.05	9.0 ± 0.10
Na, m.e.	144.6 ± 1.1	146.1 ± 1.9	146.1 ± 1.3	145.3 ± 1.6
K, m.e.	3.5 ± 0.23	3.1 ± 0.16	4.2 ± 0.18	3.5 ± 0.31

$$\epsilon = \sqrt{\frac{\Sigma x^2 - [\bar{x}\,(\Sigma x)]}{n\,(n-1)}}\,.$$

DCA caused a significant increase in systolic, diastolic, and mean blood pressures, an effect which was neither opposed nor increased by growth hormone. Growth hormone alone did not alter the blood pressure. The moderate increase in heart weight caused by DCA was absent in the group receiving growth hormone as well.

DCA tended to increase kidney weight, as usual, while growth hormone tended to decrease it slightly. The kidneys of animals given growth hormone together with DCA were not as large as those of rats receiving DCA alone. DCA clearly decreased adrenal weight, growth hormone increased it.

Both DCA and growth hormone produced a slight elevation in plasma sodium, although this is too small to be statistically significant. DCA tended to reduce plasma potassium, as usual, while growth hormone tended to increase this value.

Experiment No. 2

Thirty-two male albino rats weighing an average of 74 gm. were divided into four matched groups of eight. Group 1 served as untreated controls, group 2 received 2 mgm. of Compound F daily by subcutaneous injection, group 3 received 1 mgm. of growth hormone daily as described above, and group 4 received both Compound F and growth hormone. The experiment was terminated on the 31st day. Table II presents the pertinent data.

TABLE II

	Group			
	Control	Compound F	Growth hormone	Compound F + growth hormone
Initial body wt., gm.	76 ± 3	75 ± 4	74 ± 3	73 ± 3
Final body wt., gm.	227 ± 6	132 ± 6	210 ± 10	133 ± 6
Direct aortic blood pressure, mm.Hg				
Systolic	114 ± 6	127 ± 9 $p < 0.2$	114 ± 6	139 ± 6
Diastolic	62 ± 4	88 ± 6 $p < 0.01$	76 ± 6	95 ± 8
Mean	91 ± 4	108 ± 9 $p < 0.1$	99 ± 5	116 ± 7
Kidney wt., mgm./100 cm.^{2}	493 ± 10	590 ± 21	447 ± 13	548 ± 13
Heart wt., mgm./100 cm.^{2}	231 ± 7	227 ± 8	220 ± 8	226 ± 0.9
Adrenal wt., mgm./100 cm.^{2}	7.2 ± 0.28	2.9 ± 0.12	10.6 ± 0.40	3.0 ± 0.16
Na, m.e.	139.4 ± 0.78	137.5 ± 1.12	139.5 ± 0.46	138.2 ± 1.04
K, m.e.	3.4 ± 0.06	3.8 ± 0.23	3.0 ± 0.18	3.5 ± 0.19

Again growth hormone produced no change in body weight and, indeed, failed to offset the growth inhibiting effect of Compound F. As expected, Compound F produced a moderate increase in systolic and mean blood pressures and a significant rise in diastolic pressure, while, in contrast, growth hormone, given alone, was without effect. Given with Compound F, however, there was a distinct tendency, although not a statistically significant one, for growth hormone to increase the hypertensive effect of the steroid. Electrolyte values were not altered by either hormone in this experiment.

Experiment No. 3

Since in our work with Compound F the absence of the adrenal gland was found to intensify the hypertensive effects of the steroid (5), growth hormone was now tested in the adrenalectomized animal.

Twenty-four male albino rats weighing an average of 125 gm. were divided into three equal groups. Group 1 served as untreated controls, group 2 received 1 mgm. of growth hormone daily as described, and group 3 was bilaterally adrenalectomized five days before the experiment and then given growth hormone in the same way as group 2. The adrenalectomized rats were maintained on saline during the five days between operation and the start of the experiment. The experiment was terminated on the 15th day. Table III presents the pertinent data.

The most striking single feature of this experiment was the large mortality rate observed in the adrenalectomized animals given growth hormone. This finding is not haphazard, since this experiment is the second one carried out under the same conditions; in the first experiment all of the adrenalectomized

TABLE III

	Group		
	Control	Growth hormone	Adrenalectomy + growth hormone
Initial body wt., gm.	129 ± 2	131 ± 2	115 ± 3
Final body wt., gm.	193 ± 7	208 ± 4	179 ± 13 5 of 8 died
Direct aortic blood pressure, mm.Hg			
Systolic	105 ± 8	112 ± 5	114 ± 10
Diastolic	57 ± 5	62 ± 4	73 ± 9
Mean	78 ± 6	84 ± 4	87 ± 3
Kidney wt., mgm./100 cm.^{2}	469 ± 12	473 ± 10	421 ± 23
Heart wt., mgm./100 cm.^{2}	205 ± 7	203 ± 5	228 ± 13
Adrenal wt., mgm./100 cm.^{2}	6.3 ± 0.2	7.1 ± 0.14	—
Na, m.e.	145.9 ± 0.96	138.8 ± 1.5	141.6 ± 0.52
K, m.e.	4.1 ± 0.11	4.3 ± 0.06	5.0 ± 0.67

animals succumbed. The only tentative explanation possible from the data which we obtained is to be found in the low plasma sodium values obtained in growth hormone treated animals. If growth hormone favors sodium loss in adrenalectomized rats it is not to be wondered that the animals fail to survive. Other than this, growth hormone produced no notable alterations in the adrenalectomized rats.

Discussion

In these experiments, growth hormone produced no notable change in blood pressure or kidney size, the two major indices in the rat of a cardiovascular–renal effect. These results are at variance with those of Selye and his co-workers. It might be thought that perhaps our differences are due to the lower dose used in these experiments (1 mgm. per day as compared with 3–6 mgm. per day). Selye had, however, reported a synergism between DCA and growth hormone (8) so that even our lower dose of growth hormone should have aggravated the DCA effect when the two were given together. Actually, growth hormone in our hands failed to affect the severity of DCA hypertension and only slightly increased the hypertensive effect of Compound F. The other possible explanation for our results lies perhaps in the preparation used. It is possible that "impurities" such as thyrotrophic or lactogenic hormones may be of particular moment.

Summary

Growth hormone was administered to rats at a dose level of 1 mgm. per day for periods ranging from three to four weeks.

At this dose level growth hormone did not affect either blood pressure or kidney size when given alone, or with DCA, or with Compound F, or in the adrenalectomized animal.

Growth hormone causes a high mortality rate in adrenalectomized rats.

Acknowledgments

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THE GLYCEROL DEHYDROGENASES OF *PSEUDOMONAS SALINARIA*, *VIBRIO COSTICOLUS*, AND *ESCHERICHIA COLI* IN RELATION TO BACTERIAL HALOPHILISM[1]

By R. M. Baxter and N. E. Gibbons

Abstract

Glycerol dehydrogenases from the extremely halophilic *Pseudomonas salinaria* and the moderately halophilic *Vibrio costicolus* are described and compared with the corresponding enzyme from the nonhalophilic *Escherichia coli*. The properties of all three enzymes are similar except their responses to salt concentration. The enzymes from *E. coli* and *V. costicolus* are most active at sodium chloride concentrations of about 0.25 *M* and 0.5 *M* respectively; that from *P. salinaria* is not only most active in the presence of 1.5 *M* NaCl but is irreversibly inactivated in the absence of salt. All three enzymes are more active in the presence of potassium chloride than of sodium chloride at any given molar concentration. These results suggest that the extremely halophilic bacteria contain high concentrations of salt and that their enzymes function maximally at these high concentrations. In contrast the moderately halophilic organisms contain relatively little salt and their enzymes are more comparable with those of nonhalophiles.

The phenomenon of halophilism in bacteria has been attributed to resistance of the bacterial proteins to salt (9) or to passive or active mechanisms maintaining the intracellular salt concentration at a level below that of the medium. Evidence of an active mechanism has been reported for the moderately halophilic *Micrococcus halodenitrificans* which grows at salt concentrations ranging from 2 to 23%, the optimum being about 4% (15). Intact cells of this organism reduced nitrite most rapidly at a sodium chloride concentration of 2.2% (w/v), while the optimal salt concentration for the nitrite reductase in cell-free extracts was about 0.9% (17). Whether a similar mechanism occurs in the extremely halophilic red-pigmented organisms was not known. A comparison has therefore been made of some of the properties, particularly the responses to salts, of the glycerol dehydrogenases of three organisms: *Pseudomonas salinaria*, an extreme halophile, requiring not less than 15% sodium chloride in the medium; *Vibrio costicolus*,* a moderate halophile growing in salt concentrations ranging from about 1–20% (7,13); and *Escherichia coli*, a nonhalophilic organism.

Materials and Methods

The strain of *Pseudomonas salinaria* used in this investigation was originally isolated by Lochhead (10) from salted hides. Cells were grown in liquid medium of the following composition: casamino acids (Difco) 5 gm., yeast extract (Difco) 5 gm., proteose-peptone (Difco) 5 gm., trisodium citrate

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* *It has been brought to our attention that the suffix* -colus *is etymologically incorrect and that the specific name should be* costicola. *The original spelling is retained in this paper pending a decision by the editors of Bergey's Manual.*

3 gm., potassium chloride 2 gm., $MgSO_4 . 7H_2O$ 20 gm., sodium chloride 200 gm., distilled water to 1000 ml. Cultures were incubated at 30° C. with continuous aeration for seven or eight days and the cells harvested in a Sharples centrifuge, washed twice with 20% NaCl, and resuspended in 20% NaCl.

The strain of *Vibrio costicolus* used was originally isolated in this laboratory from a bacon curing brine. It appears to be identical with the organism originally described by Smith (18). Cells were grown in a medium containing proteose-peptone (Difco) 5 gm., tryptone (Difco) 5 gm., sodium chloride 40 gm., and distilled water to 1000 ml. After about 24 hr. at room temperature the cells were harvested in the Sharples, washed twice with 4% NaCl and resuspended in 4% NaCl.

Escherichia coli (N.R.C. No. 482) was grown in tryptone–glucose–yeast extract broth (1). After 16–18 hr. incubation at 30° C. the cells were harvested in the Sharples, washed twice, and resuspended in distilled water.

Cell-free extracts were prepared in a "Raytheon" 10 kc. sonic vibrator. With the output current set 1–1.2 amp., cells of *P. salinaria* were disrupted in 10 min., those of *V. costicolus* in 20 min., and those of *E. coli* in 40 min. The extracts were centrifuged in an M.S.E. high speed centrifuge at 12,000–13,000 r.p.m. (approx. 16,000 g.) for 30–40 min., and the supernatants decanted and stored at 4–5° C. under toluene. Very little cellular debris was obtained from the *P. salinaria* extract, but on storage a flocculent precipitate gradually appeared, and was removed by centrifugation from time to time, without causing loss of activity. All the preparations retained considerable enzyme activity even after two or three months storage at 4–5° C. These crude extracts were used in most of the experiments.

Partial purification of the *P. salinaria* and *E. coli* glycerol dehydrogenases was achieved by the method described by Asnis and Brodie (1) for the *E. coli* enzyme, involving removal of other proteins by heat denaturation at 60° C. for 90 min. and precipitation of the enzyme from the supernatant by saturation with ammonium sulphate. To prevent inactivation the *P. salinaria* enzyme was dialyzed against 20% NaCl rather than distilled water. An attempt to purify the *V. costicolus* enzyme was unsuccessful.

Enzyme activities were usually measured manometrically at 35° C., using the ferricyanide technique of Quastel and Wheatley (11). The ferricyanide solution used was prepared by mixing 5 ml. of 20% $K_3Fe(CN)_6$ or $Na_3Fe(CN)_6$ and 1 ml. of 0.16 *M* $NaHCO_3$. The buffer was 0.025 *M* $NaHCO_3$ equilibrated with 5% CO_2 in nitrogen. The pH under these conditions (about 7.5) was much below the optimum for the enzymes (see below) but the method proved extremely satisfactory.

The vessel constants were corrected where necessary for the effect of salt on the solubility of carbon dioxide according to the data of Harned and Davis (8).

The manometric method could not be used to determine the effect of pH or the product of oxidation, and here the reaction was followed in the Beckman

spectrophotometer by measuring the increase in optical density at 340 mμ caused by reduced DPN. Phosphate buffer was used for pH values below 8, and glycine – sodium hydroxide buffer for higher values.

Diphosphopyridine nucleotide (DPN) of 55 or 65% stated purity and triphosphopyridine nucleotide (TPN) of 65% purity were obtained from the Sigma Chemical Co.

Results

Properties of the Enzymes

Some of the properties of the three glycerol dehydrogenases are summarized in Table I and discussed below.

TABLE I

PROPERTIES OF GLYCEROL DEHYDROGENASES

	Organism		
	Pseudomonas salinaria	*Vibrio costicolus*	*Escherichia coli*[2]
Coenzyme	DPN[1] TPN not active	DPN TPN not tested	DPN TPN not active
Substrates oxidized	Glycerol	L-2,3-butanediol > 1,2-propanediol > ethanediol > glycerol	Glycerol
Substrates not oxidized	*d*-Sorbitol, *d*-mannitol, ethanol, L-2,3-butanediol	*d*-Sorbitol	*d*-Sorbitol, *d*-mannitol, erythritol, ethanol, L-2,3-butanediol*
pH Optimum	10	10	10
K_S[3]	1×10^{-1} *M*	2×10^{-1} *M*	1.07×10^{-2} *M* 2×10^{-1} *M**
K_{DPN}[3]	5×10^{-4} *M*	1×10^{-4} *M*	2.6×10^{-4} *M*
Product	DHA[1]	Not determined	DHA
Heat stability	Resists 60° C. for 90 min. (in 20% salt)	Largely inactivated at 60° C. for 90 min. (in 4% salt)	Resists 60° C. for 90 min. (in H_2O)

[1] *DPN—diphosphopyridine nucleotide; TPN—triphosphopyridine nucleotide; DHA—dihydroxyacetone.*

[2] *Data from Asnis and Brodie (1) except those marked*.*

[3] *In the present investigation K_S was determined at a DPN concentration of 0.001 M and K_{DPN} at a glycerol concentration of 1.0 M.*

Coenzyme specificity.—The *P. salinaria* enzyme has a specific DPN requirement (Fig. 1); TPN did not replace DPN. The *E. coli* enzyme has a similar DPN specificity (1). The enzyme from *V. costicolus* also requires DPN; TPN was not tested.

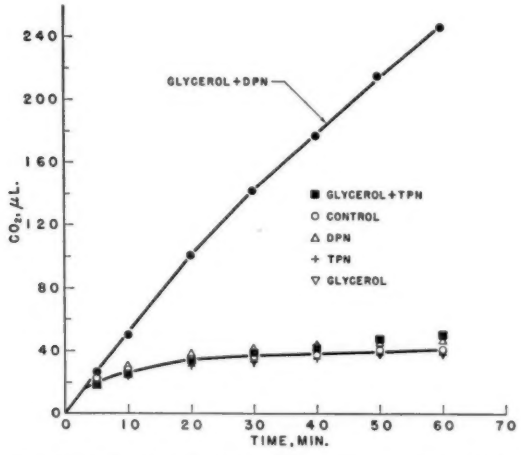

FIG. 1. Requirement of glycerol dehydrogenase of *P. salinaria* for DPN. Contents of vessels: as in "Methods"; also NaCl, 1.7 *M* (10%); $K_3Fe(CN)_6$ (20%), 0.1 ml.; glycerol (when present) 1.0 *M*; DPN or TPN (when present) 0.001 *M*; reaction started by tipping 0.2 ml. cell-free extract from side-arm. Final volume 3.2 ml.

Substrate specificity.—The *P. salinaria* and *E. coli* enzymes appeared to be specific for glycerol. However extracts of *V. costicolus*, in the presence of DPN, oxidized the following substrates (listed in order of decreasing rate of oxidation at 0.31 *M*): butanediol, propanediol, ethylene glycol, and glycerol. Attempts to separate glycerol dehydrogenase activity from butanediol dehydrogenase activity, or to inhibit one or the other differentially, were not successful. It is possible therefore that *V. costicolus*, in contrast to the other two organisms, contains a relatively nonspecific polyalcohol dehydrogenase. For convenience however all three enzymes will be referred to as glycerol dehydrogenases in this paper.

Effect of pH.—The activities of the enzymes of *P. salinaria* and *V. costicolus* were measured spectrophotometrically at several pH values. The responses of the two enzymes to pH were essentially the same; the activity rose gradually from zero at about pH 6 to a maximum at pH 10, then fell very sharply at pH 10.5–11. A virtually identical curve was published by Asnis and Brodie (1) for the *E. coli* enzyme.

Effect of substrate and coenzyme concentration.—The concentrations of glycerol giving half-maximum activities (K_s) were of the same order for the three enzymes. The corresponding constants for DPN (K_{DPN}) were also of the same order. The discrepancy between the K_s values for the *E. coli* enzyme obtained by Asnis and Brodie and the present authors (Table I) is probably the result of the different pH values necessitated by the different methods employed (cf. Dixon (6)). Asnis and Brodie measured activity spectrophotometrically at pH 10 while the present authors used the manometric method at pH about 7.5.

Effect of enzyme concentration.—The rate of glycerol oxidation per unit of enzyme declined as the enzyme concentration was increased with both the *E. coli* and the *P. salinaria* enzymes (Table II).

TABLE II

EFFECT OF ENZYME CONCENTRATION ON THE RATE OF GLYCEROL OXIDATION BY THE GLYCEROL DEHYDROGENASES OF *E. coli* AND *P. salinaria*

Source of enzyme	Volume of cell-free extract, ml.	CO_2 evolved in 30 min., μl.	CO_2/0.1 ml. extract/30 min., μl.
E. coli	0.05	77	154
" "	0.1	131	131
" "	0.2	228	114
P. salinaria	0.1	67	67
" "	0.2	112	56
" "	0.4	182	46

Contents of vessels: as in "Methods"; also glycerol, 1.0 M; DPN (55%) 7.9 mgm. per vessel; with P. salinaria enzyme NaCl, 3.4 M (20%). Reaction started by adding 0.2 ml. $K_3Fe(CN)_6$ from side-arm. Final volume 3.2 ml.

Product of glycerol oxidation.—Asnis and Brodie (1) identified dihydroxyacetone (DHA) colorimetrically as the product of the action of the *E. coli* enzyme. Burton and Kaplan (4) found that, in the presence of the glycerol dehydrogenase of *Aerobacter aerogenes*, the reduction of DPN (measured spectrophotometrically at 340 mμ) by glycerol was reversed more rapidly by DHA than by glyceraldehyde (GA). They concluded that the product of glycerol oxidation was DHA rather than GA. By the same method it was found that the product of glycerol oxidation by the *P. salinaria* enzyme is also DHA. The product of the *V. costicolus* enzyme was not determined, but it seems probable that it too produces DHA.

Effects of Salts on the Enzymes

Effects of sodium and potassium chlorides.—The glycerol dehydrogenase from *E. coli* was slightly stimulated by low concentrations of either sodium or potassium chloride, but was progressively inhibited by higher concentrations of both salts, sodium being rather less stimulatory and more inhibitory than potassium (Fig. 2). The enzyme from *V. costicolus* reacted to sodium chloride in a similar manner but was stimulated much more strongly by potassium than by sodium at the lower concentrations tested. Above about 1.5 *M* potassium inhibited this enzyme as well. The response to either salt was the same whether the organisms had been grown in 4% or 15% NaCl (Fig. 3). The similarity of the effect of sodium on these two enzymes is more readily seen by a comparison of their responses to sodium chloride in the presence of approximately optimal concentrations of potassium chloride (0.25 *M* for the *E. coli* enzyme and 0.5 *M* for the *V. costicolus* enzyme (Fig. 4)). The

similarity of the responses gives further support to the theory of Robinson *et al.* (17) that the enzymes of moderately halophilic organisms are not much more resistant to salts than those of ordinary bacteria.

The *P. salinaria* enzyme on the other hand reacted quite differently to salts (Fig. 5). This enzyme was inactive at low salt concentrations but was still active at salt concentrations much below the minimum at which *P. salinaria* grows, or even remains viable (5). With increasing concentrations of

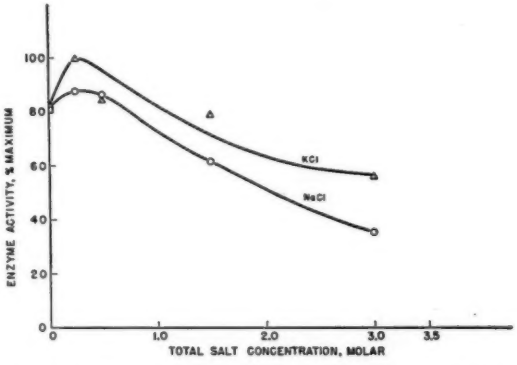

FIG. 2. Effect of sodium chloride and potassium chloride on the activity of the glycerol dehydrogenase of *E. coli*.

Contents of vessels: as in "Methods"; also glycerol, 0.4 *M*; DPN (65%) 3 mgm.; cell-free extract, 0.1 ml.; salts as indicated; reaction started by tipping 0.1 ml. $Na_3Fe(CN)_6$ from side-arm. Final volume 1.6 ml. Activity measured over period 5–35 min. after tipping.

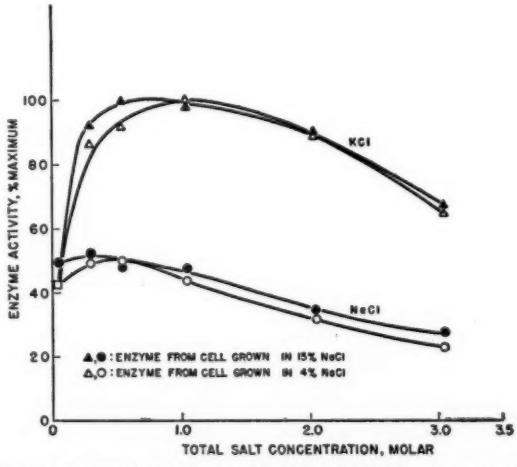

FIG. 3. Effect of sodium chloride and potassium chloride on the activity of the glycerol dehydrogenase of *V. costicolus*. Both extracts prepared and stored in 4% NaCl. (Details as in caption to Fig. 2.)

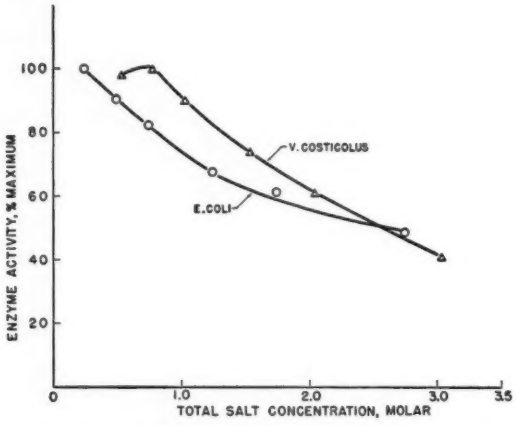

FIG. 4. Effect of sodium chloride on the activity of the glycerol dehydrogenases of *E. coli* and *V. costicolus* in the presence of optimal concentrations of potassium chloride, i.e. with *E. coli* extract, 0.25 *M*; with *V. costicolus* extract, 0.5 *M*. Average of two experiments. (Other details as in caption to Fig. 2.)

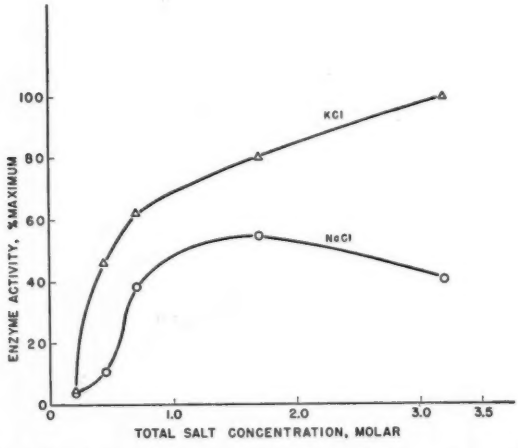

FIG. 5. Effect of sodium chloride and potassium chloride on the activity of glycerol dehydrogenase of *P. salinaria*. (Details as in caption to Fig. 2.)

potassium chloride the activity increased rapidly, and was found to be still increasing at a concentration of 4 *M*. With increasing concentrations of sodium chloride, the increase in activity was not as rapid or as great, and there was a decline in activity at concentrations above 1.5 *M*.

The responses of the *P. salinaria* and *E. coli* enzyme to sodium chloride were not changed by purification (Table III).

TABLE III

EFFECT OF NaCl ON CRUDE AND SEMIPURIFIED GLYCEROL DEHYDROGENASES OF *E. coli* AND *P. salinaria*

Salt conc. (gm./100 ml.)	Activity, % of maximum			
	E. coli enzyme		*P. salinaria* enzyme	
	Crude	Semipurified	Crude	Semipurified
0	100	100	—	—
1.25	—	—	2.2	6.9
2	—	—	6.1	12.4
5	73	70	86	79.4
10	55	47	100	100
15	39	35	93	91.6
20	30	32	82	76.4

Contents of vessels: as in "Methods"; also glycerol, 0.5 M; DPN (55%), 3.6 mgm.; cell-free extracts: E. coli, crude, 0.1 ml., semipurified, 0.4 ml.; P. salinaria, crude, 0.1 ml., semipurified, 0.2 ml.; reaction started by tipping 0.2 ml. $K_3Fe(CN)_6$ from side-arm. Final volume 3.2 ml.

It seemed possible that the differing salt responses of the *P. salinaria* and *E. coli* enzymes might be the result of an influence of salt on an interaction between the enzymes and some inhibitory or activating factor also present in the extracts. Experiments designed to determine whether such a factor was present gave no evidence of any interaction between the two extracts (Table IV). The data in the first line indicate that at low salt concentrations

TABLE IV

ACTIVITY OF MIXTURES OF GLYCEROL DEHYDROGENASES OF *E. coli* AND *P. salinaria*

Salt concentration, *M*	CO_2 in 30 min., μl.			
	E. coli extract 0.1 ml.	*P. salinaria* extract 0.2 ml.	*E. coli* extract 0.1 ml. + *P. salinaria* extract 0.2 ml.	*E. coli* extract 0.02 ml. + *P. salinaria* extract 0.1 ml.
0.21	94.5	9.3	105 (101%)[1]	60.7 (117%)[2]
3.4	36.9	60.7	82.2 (85%)[1]	47.8 (99%)[2]

Single enzyme determinations based on average of two experiments. Combined enzyme determinations based on single experiments.

[1] *Activity expressed as % of sum of separate activities.*

[2] *Activity expressed as % of average of separate activities.*

Contents of vessels: as in "Methods"; also glycerol, 1.0 M; other components as indicated. Reaction started by adding 0.2 ml. $K_3Fe(CN)_6$ from side-arm. Final volume 3.2 ml.

the presence of *P. salinaria* extract did not lower the activity of the *E. coli* enzyme, and conversely the presence of *E. coli* extract had no protective influence on the *P. salinaria* enzyme. Likewise at high salt concentrations (last line) there was no evidence of stimulation of the *E. coli* enzyme by *P. salinaria* extract, or inhibition of the *P. salinaria* enzyme by *E. coli* extract. The activities of mixtures of the two enzymes are not quite additive with respect to the activities of the enzymes when acting separately because of the nonlinear relationship between activity and enzyme concentration shown above.

Effect of other salts.—The other alkali metals were tested (as chlorides) to determine the specificity of these effects. With lithium chloride the activity of the *P. salinaria* enzyme was about the same as with sodium chloride at concentrations up to 0.5 *M*, but decreased at higher concentrations; all three enzymes showed negligible activity in 3 *M* LiCl. Different samples of rubidium chloride and cesium chloride activated the *P. salinaria* enzyme to different degrees, perhaps because of different amounts of impurities. It appeared, however, that the activating effects of these two salts were similar to the effect of potassium chloride. With the *V. costicolus* enzyme, rubidium and cesium had about the same effect as potassium. With the *E. coli* enzyme, the inhibition by rubidium and cesium fell in the same range as that of potassium and sodium.

The nature of the anion appears to be of less importance than the nature of the cation. Equimolar concentrations of potassium chloride, bromide, or nitrate produced about the same response with the enzymes from *E. coli* and *V. costicolus*. With the *P. salinaria* enzyme, the activity with potassium bromide or nitrate was about 40% less than with the same concentration (3 *M*) of potassium chloride. The effect of salts on this enzyme appears to be ionic rather than merely osmotic, since urea (at 6.5 *M*) did not activate the enzyme. Moreover since the Michaelis constant of the system is very high, glycerol concentrations up to 1.0 *M* were used, but even at this concentration of substrate the enzyme was inactive in the absence of salts.

Nature of the salt responses of the enzymes.—It appears that the response of the *P. salinaria* enzyme to salts results from two opposing effects. On the one hand, salts are necessary for the activity of the enzyme; the inactivation that occurred in the absence of salts could not be reversed by restoring either sodium or potassium (Table V). This inactivation was not accompanied by precipitation of the enzyme. On the other hand, high concentrations of certain salts (sodium chloride or lithium chloride) produced an inhibition which, with sodium at least, was reversible, since the activity of extracts stored in 20% (3.4 *M*) NaCl was always greater in about 1.5 *M* NaCl than in higher concentrations. The relative magnitudes of these two effects at any given salt concentration depended on the nature of the cation, and to a lesser extent, the anion.

Inactivation of the *P. salinaria* enzyme by dilution is not instantaneous but occurs at a rate dependent on the salt concentration (Fig. 6). To measure

TABLE V

IRREVERSIBILITY OF THE INACTIVATION OF GLYCEROL DEHYDROGENASE OF *P. salinaria* CAUSED BY DILUTION

Extract diluted with:	NaCl concentration in Warburg vessel	KCl concentration in Warburg vessel	CO_2 evolved in 60 min., μl.
H_2O	2.05 *M*	—	2.3
H_2O	0.05 *M*	2.0 *M*	17.3
20% NaCl	2.0 *M*	—	104
20% NaCl	0.5 *M*	1.5 *M*	163

One milliliter of cell-free extract in 20% NaCl diluted to 10 ml. with H_2O or 20% NaCl, let stand at room temperature for 21 hr., then tested in Warburg apparatus; glycerol 0.6 M; DPN (65%), 3.0 mgm. per vessel; NaCl or KCl as indicated. Reaction started by adding 0.2 ml. $K_3Fe(CN)_6$ from side-arm. Final volume 3.2 ml.

the activity from the moment of dilution, the enzyme (in 20% NaCl) was placed in the side-arms of the Warburg flasks and tipped into the main compartments containing the other components of the system and varying amounts of salts. The figures on the curves represent the concentration of salt in the

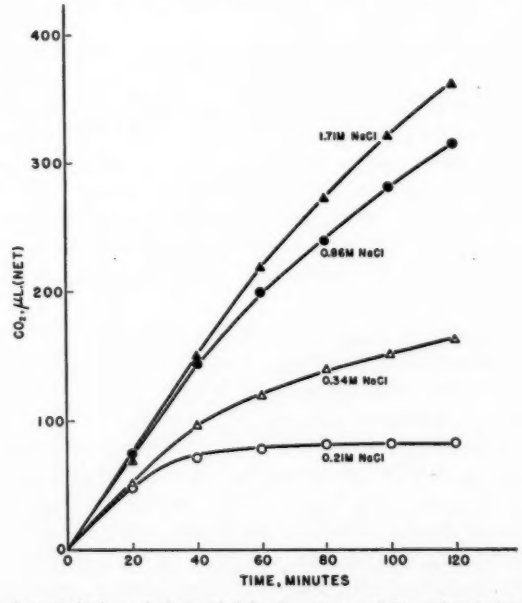

FIG. 6. Inactiviation of glycerol dehydrogenase of *P. salinaria* by dilution.

Contents of vessels: as in "Methods"; also glycerol, 0.5 *M*; DPN (55%), 3.6 mgm.; $K_3Fe(CN)_6$ solution, 0.1 ml.; reaction started by tipping 0.2 ml. cell-free extract from side-arm. Final volume 3.2 ml.

vessels after mixing. The activity of the *P. salinaria* enzyme under constant conditions of temperature, pH, etc., is therefore dependent on three factors: (i) the nature and (ii) concentration of any salts that may be present, and (iii) the length of time the enzyme has been exposed to the salts.

The inhibition of the *E. coli* enzyme by high concentrations of sodium chloride is reversible, and appears to be similar to the high-concentration inhibition of the *P. salinaria* enzyme.

Discussion

The results presented indicate that the glycerol dehydrogenase of *P. salinaria* is much more resistant to salts than the analogous enzymes from *E. coli* and *V. costicolus*. If all the enzymes of each organism behave in the same way as the glycerol dehydrogenases, these findings strongly suggest that two fundamentally different mechanisms exist for adaptation to a halophilic existence.

The first type of adaptation is at the cellular level, and consists of some mechanism, probably energy dependent, that maintains the intracellular salt concentration at a level considerably below that of the environment. This appears to be the mechanism employed by those organisms which can grow over a relatively wide range of salt concentrations, e.g. *M. halodenitrificans* (17) and *V. costicolus* (7,14,18). A similar process seems to occur in some multicellular halophiles such as *Artemia salina* (21), and the larvae of *Aedes detritus* (3).

The second mechanism occurs in *P. salinaria*, and probably in the red halophiles in general. These organisms grow over a more restricted, and very high, range of salt concentrations. Here the adaptation appears to be at the molecular level and to involve some change in the enzyme molecule which not only makes it most active in the presence of high concentrations of salt but also renders it inactive in the absence of salts. This assumption is supported by the work of Robinson and Katznelson (16) showing that the response to salt of the aspartate–glutamate transaminase of *P. salinaria* is similar to that of the glycerol dehydrogenase. The basis of this requirement for salt is not known.

There are a number of reports in the literature of salt effects on enzymes which at least superficially resemble the salt responses of the *P. salinaria* enzyme. For example, the inactivation of enzymes by the removal of salts has been reported by Straub (20) and Baranowski (2). It seems probable, however, that this resemblance is more apparent than real, since both Straub and Baranowski used crystalline enzymes which have often been reported to be rather unstable, and the inactivations occurred at salt concentrations much lower than that at which the *P. salinaria* enzyme is inactivated.

Another observation of possible relevance is that of Riley, Hobby, and Burke (12) who observed that low concentrations of salts activated mammalian cytochrome oxidase by preventing combination of the enzyme with a naturally-occurring inhibitor. It seemed possible that some such mechanism might be involved in the present instance, but further investigation has

rendered this unlikely. The combination of the enzyme with such an inhibitor should be reversible, but the inactivation caused by dilution was not reversible. Furthermore, it would seem almost certain that the ratio of enzyme to inhibitor would be altered on purification of the enzyme, but the response of the *E. coli* and *P. salinaria* enzymes to sodium chloride was found to be essentially the same whether crude or partially purified preparations were used. The most direct evidence against this possibility is that the activities of the *E. coli* and *P. salinaria* enzyme are not influenced by the presence of extracts of the other organism, either in the presence or absence of salt.

Ingram (9) suggested that enzymes of halophiles "may perhaps be involved in smaller molecular aggregates than in the case of normal cells" and so be more resistant to salting out. However there is evidence (19) that enzymes attached to large molecules are more resistant to heat inactivation than if not so attached. The enzymes in question from both *P. salinaria* and *E. coli* are unusually heat stable.

Although similar in many respects, the glycerol dehydrogenases of *E. coli* and *P. salinaria* differ strikingly in their sensitivity to salts. From the above discussion, it seems probable that this difference is the result of unknown modifications in the structure of the enzyme proteins. It also seems probable that the instability of the glycerol dehydrogenase of *P. salinaria* at low salt concentrations is closely linked with the obligate halophilism of the red halophilic bacteria.

Acknowledgment

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FACTORS AFFECTING THE BIOASSAY OF 11-OXYCORTICOSTEROIDS IN ADRENALECTOMIZED RATS[1]

By N. R. Stephenson

Abstract

Total sugar in a protein-free liver hydrolyzate, determined by copper reduction, provides an acceptable criterion for estimating the response to graded doses of 11-oxycorticosteroids. Total carbohydrate in the liver, measured by the anthrone reagent, may also be used for this purpose. The glycogenic response to adrenal corticoids of fasting adrenalectomized male rats of the Wistar strain, weighing 40 to 50 gm., is increased by the administration of glucose, but appears to be depressed by ethanol. Although the precision of the assay is not influenced unfavorably by using the rats on the day following adrenalectomy, the mortality rate of the adrenalectomized animals is reduced by this procedure.

The use of liver glycogen deposition as the criterion of the response to 11-oxycorticosteroids by fasting adrenalectomized rats and mice has been the basis of several bioassay procedures (3,4,7,8,9,14). Shorr (12) suggested that less variable results would be obtained in such assays if total fermentable sugars rather than liver glycogen were determined in the liver. Eggleston, Johnston, and Dobriner (4) applied this suggestion to the bioassay of adrenal cortical steroids using adrenalectomized mice and reported that the use of fermentable sugars in the liver as the response improved the precision of the assay by decreasing the mean standard deviation and increasing the slope of the log dose–response line.

Since the determination of fermentable sugar in the liver is time-consuming and therefore not particularly suitable for routine use, it was decided to investigate the possibility of simplifying the procedure by omitting the fermentation step and using total reducing sugar in the liver as the criterion of the response to 11-oxycorticosteroids. The total carbohydrate in liver was measured in protein-free hydrolyzates by two procedures: (i) substances which reduce copper, as determined by the Nelson colorimetric method (6), and (ii) substances which react with the anthrone reagent to give an absorption maximum at 625 mμ (5,10,11,15).

A study was made of various functions of the response to 11-oxycorticosteroids which could be applied to the assay of pharmaceutical preparations of adrenal cortical extracts. Other factors influencing the assay were also examined, including the effect of (*a*) the vehicle or solvent used for the corticoid material, (*b*) the administration of glucose during the injection period, and (*c*) the time elapsing between adrenalectomy and the injections of the hormone preparation.

[1] *Manuscript received December 31, 1953.*

Contribution from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa.

Experimental

Male rats of the Wistar strain, approximately 30 days of age and weighing 40 to 50 gm., were bilaterally adrenalectomized under ether anesthesia, and were kept at 26 ± 1° C. following the operation. The regular stock diet* was used with 1% sodium chloride as the drinking water. For the first half day following adrenalectomy, 5% glucose was added to the saline to assist in the recovery from postoperative shock. At 5 P.M. on the day before the test, all food was removed from the cages and the rats were weighed and distributed at random among the dosage groups. Since the response to the hormone was influenced by the body weight, the total weight of the rats within each treatment group was equalized by appropriate exchange after the original random selection. The next morning, the 11-oxycorticosteroid preparation was administered subcutaneously in four divided injections at 9 A.M., 10.30 A.M., 12 noon, and 1.30 P.M. In addition, each rat received a concomitant subcutaneous injection of 25 mgm of glucose, making a total of 100 mgm. administered over the whole injection period. At 3.30 P.M., the rats were anesthetized with sodium amytal (0.4 ml. of a 1.8% solution) given intraperitoneally. When both liver sugar and glycogen were to be determined, one lobe of the liver was removed *in situ*, blotted quickly on filter paper, weighed, and then dropped into a 30% solution of potassium hydroxide. The glycogen in this sample was estimated by a modification of the indirect method of Seifter *et al.* (10) employing the anthrone reagent. The remainder of the liver was removed, blotted on filter paper, weighed, and dropped into a 50 ml. graduated pyrex centrifuge tube containing 10 ml. of 1 *N* sulphuric acid. Hydrolysis of the carbohydrate in the sample was accomplished by heating the tube for 30 min. in a bath of boiling water, breaking up the liver with a glass rod, then autoclaving at 15 lb. pressure for 15 to 20 min. After cooling, the hydrolyzate was neutralized, adjusted to a volume of 30 ml. with water, and a 2 ml. aliquot was deproteinized by means of zinc hydroxide (13). The glucose equivalent of the protein-free hydrolyzate was determined both by copper reduction using the Nelson colorimetric method (6), and by the anthrone reagent. Fig. 1 illustrates spectral transmission curves for the color produced when anthrone in sulphuric acid is allowed to react with glucose and with typical deproteinized liver hydrolyzates. A 5 ml. aliquot of the solution containing the hydrolyzed carbohydrate was kept cold while it was mixed with 10 ml. of the anthrone reagent. This reaction mixture was then heated in a bath of boiling water for 10 min. to allow the chromogen to develop. In Fig. 1 the maximum observed at 625 mμ is due to glucose or its equivalent, while the other maximum at approximately 500 mμ can probably be attributed to the presence of amino acids such as tryptophane (10,11) in the deproteinized liver hydrolyzate. Consequently a 620 filter was used in the Evelyn photoelectric colorimeter for determining the optical density of the chromogen. Glucose was employed as the reference standard for both the copper reduction procedure and the anthrone method.

* *Master Fox Cubes—Toronto Elevators Ltd.*

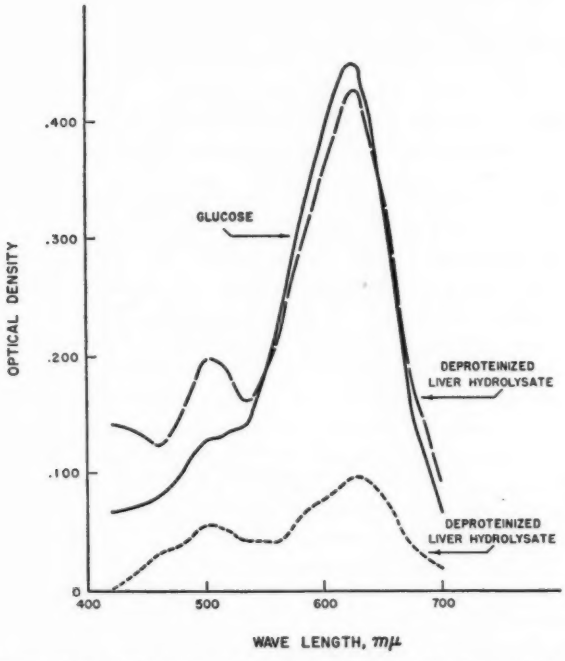

FIG. 1. Comparison of the spectral absorption curves of the chromogens produced by (*a*) glucose, and (*b*) protein-free liver hydrolyzates, with the anthrone reagent.

Results and Discussion

Comparison of Liver Reducing Sugar and Glycogen

Table I shows that the mean differences between the reducing sugar values and those for glycogen in the same liver samples, were practically constant for the doses of cortisone acetate used. A similar difference was observed when an adrenal cortical extract in oil was employed instead of cortisone acetate. Accordingly, total reducing sugar in a hydrolyzate of liver tissue provided an acceptable criterion of the response to 11-oxycorticosteroids in adrenalectomized 30-day-old male rats.

The glucose equivalent of the carbohydrate in the deproteinized liver hydrolyzates, determined by the copper reduction procedure, was compared with that estimated by the anthrone method. Table II indicates that the difference between these two values was not constant over the dose range of cortisone acetate employed but was inclined to decrease as the dose of the 11-oxycorticosteroid was increased. The difference observed at the 100 μgm. dose of cortisone acetate was significantly smaller than that found at the 25 μgm. dose. Eggleston *et al.* (4) reported that fermentable sugar provided a steeper slope than glycogen when it was employed as the criterion of the

TABLE I

COMPARISON OF REDUCING SUGAR AND GLYCOGEN FOUND IN THE LIVER OF ADRENALECTOMIZED IMMATURE MALE RATS AFTER THE SUBCUTANEOUS INJECTION OF CORTISONE ACETATE

No. of rats	Dose of cortisone acetate, μgm.	Glycogen per gm. liver, mgm.	Reducing sugar per gm. liver, mgm.	Difference, mgm.
8	0	0.35 ± 0.04*	4.01 ± 0.17*	3.66 ± 0.16*
10	25	1.09 ± 0.28	5.22 ± 0.40	4.13 ± 0.26
10	50	2.56 ± 0.50	7.20 ± 0.69	4.64 ± 0.41
10	100	3.95 ± 0.77	8.41 ± 0.73	4.46 ± 0.33
9	200	5.96 ± 0.64	10.48 ± 0.75	4.52 ± 0.64
9	A.C.E.**	10.06 ± 1.03	14.11 ± 0.69	4.05 ± 0.72

* *Standard error of the mean.*
** *S. S. Lipo Adrenal Cortex Extract 0.2 ml.* (*Upjohn & Co.*).

response for the calculation of the log dose–response line. Possibly liver carbohydrate as determined by the anthrone method is related to the fermentable sugar present in the liver. This observation is supported by the fact that when the anthrone reagent was used to measure both the glycogen and the carbohydrate present in the liver, the coefficient of variation† of the former was approximately twice that of the latter. Consequently the use of liver carbohydrate as the response, like the fermentable sugar, tended to reduce the standard deviation of the assay.

TABLE II

COMPARISON OF THE GLUCOSE EQUIVALENTS DETERMINED BY COPPER REDUCTION AND BY THE ANTHRONE REAGENT IN PROTEIN-FREE LIVER HYDROLYZATES AFTER THE ADMINISTRATION OF CORTISONE ACETATE

No. of rats	Dose of cortisone acetate, μgm.	Glucose equivalent per gm. of liver		
		Copper reduction, mgm.	Anthrone reagent, mgm.	Difference, mgm.
5	0	4.02 ± 0.24*	2.60 ± 0.17*	1.42 ± 0.32*
20	25	6.72 ± 0.43	5.33 ± 0.53	1.39 ± 0.12
21	50	8.90 ± 0.36	7.81 ± 0.46	1.07 ± 0.12
21	100	9.76 ± 0.57	8.96 ± 0.72	0.80 ± 0.17

* *Standard error of the mean.*

† *The coefficient of variation* $= \frac{\textit{standard deviation} \times 100}{\textit{mean response}}$.

The Influence of Various Functions of the Response on the Precision of the Assay

Cortisone acetate dissolved in an aqueous solution containing 10% ethanol was given at four dose levels: 25, 50, 100, and 200 μgm. to adrenalectomized 30-day-old male rats. Liver reducing sugar was employed as the criterion of the response. Table III shows the data calculated in several different ways, and it is clear that all of the functions provided approximately the same order of precision. The value for λ was decreased slightly either by reducing the effect of the variation in body weight by covariance analysis (1), or by making a correction for body weight by expressing the response in terms of 100 gm. of rat. The variances at each dose level were homogenous but had a tendency to become larger as the level of the response increased.

TABLE III

THE INFLUENCE OF VARIOUS FUNCTIONS OF THE RESPONSE ON THE PRECISION OF THE ASSAY

Function of the response	Index of precision, λ	Slope, b	Coefficient of variation, c	Mean response, $\bar{y}$
Reducing sugar/liver	0.349	10.89	27.1	14.01
Reducing sugar/liver corrected for body wt. by covariance analysis	0.330	11.42	26.8	14.01
Reducing sugar/gm. of liver	0.350	6.10	26.9	7.94
Reducing sugar/100 gm. of body weight	0.314	25.56	26.6	30.11
$\frac{\text{Log reducing sugar} \times 100}{\text{Log body weight}}$	0.320	22.26	10.76	66.87
Log reducing sugar/100 gm. body weight	0.411	0.32	9.2	1.44

The coefficient of variation was reduced considerably by the function: $\frac{\text{log reducing sugar} \times 100}{\text{log body weight}}$, which also provided the most uniform variances over the entire dose range. However, since the slope of the log dose–response line was not increased proportionately to the response, the precision of the assay was not improved significantly. All of the functions examined gave a highly significant linear relationship between the logarithm of the dose and the response. The precision was not influenced significantly by expressing the reducing sugar as mgm. per gm. of liver and making no correction for the weight of the rat. Since liver weight was correlated significantly with body weight ($r = 0.86$) in these rats, body weight had an indirect effect on the response. The weight of the liver in rats at 30 days of age was found to be approximately 4% of the total body weight. For routine use in assays of 11-oxycorticosteroids, liver reducing sugar was expressed in terms of 100 gm. of rat, thus avoiding the necessity of weighing the liver prior to hydrolysis.

A study of the relationship between the body weight and the response at each dose level indicated that they were not correlated significantly. Accordingly, administration of the hormone on a weight basis probably would not improve the precision of the assay. In spite of this apparent lack of importance of body weight, it was advantageous to keep the range of body weights of the rats employed in the assays at a minimum. The index of precision was usually between 0.15 and 0.25 when the weight spread within dosage groups was approximately 5 gm. On the other hand, when the range of body weights was 10 gm. or more, λ was often between 0.30 and 0.40.

The Effect of Glucose and of the Solvent of the Steroid on the Response

Venning, Kazmin, and Bell (14) demonstrated that glucose administration resulted in a marked increase in the liver glycogen of fasting adrenalectomized mice only when they were given adrenal cortical steroids. Coté *et al.* (2) found that glucose increased the amount of glycogen deposited by 11-oxycorticosteroids in the livers of fasted adrenalectomized adult rats.

As a result of these observations, a study was made of the effect of glucose on the amount of carbohydrate deposited in the livers of immature adrenalectomized male rats after the administration of 11-oxycorticosteroids. Table IV indicates that the response to 50 μgm. of cortisone acetate in oil was significantly greater than the control values only when 100 mgm. of glucose was injected with the hormone. In addition, the difference between the glucose equivalents by copper reduction and those estimated by the anthrone reagent at the 100 mgm. dose of glucose, were smaller than those observed when lesser amounts of glucose were injected.

TABLE IV

THE EFFECT OF GLUCOSE ON THE RESPONSE OF ADRENALECTOMIZED IMMATURE MALE RATS TO CORTISONE ACETATE IN OIL

No. of rats	Glucose, mgm.	Cortisone acetate, μgm.	Glucose equivalent per gm. of liver		
			Copper reduction, mgm.	Anthrone reagent, mgm.	Difference, mgm.
10	0	0	3.66 ± 0.10*	1.60 ± 0.10*	2.06 ± 0.09*
5	100	0	4.02 ± 0.24	2.60 ± 0.17	1.42 ± 0.12
6	0	50	4.21 ± 0.18	1.96 ± 0.05	2.25 ± 0.13
6	25	50	4.66 ± 0.30	2.36 ± 0.30	2.30 ± 0.05
6	50	50	4.90 ± 0.59	2.69 ± 0.57	2.21 ± 0.07
6	100	50	9.86 ± 1.17	8.83 ± 1.50	1.03 ± 0.50

* *Standard error of the mean.*

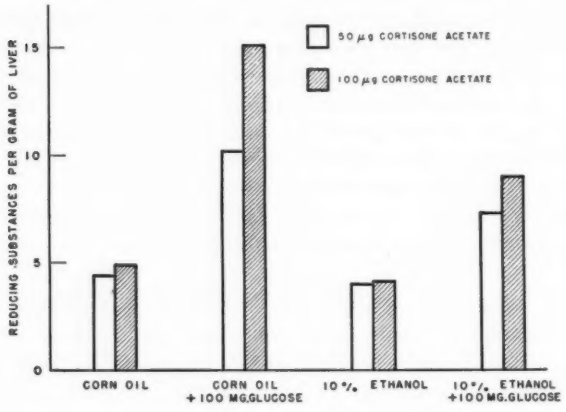

FIG. 2. The effect of glucose and of the vehicle on the biological activity of cortisone acetate.

Fig. 2 shows the results of an experiment in which liver reducing sugar was determined after cortisone acetate was given both in corn oil and in 10% ethanol with and without the concomitant administration of 100 mgm. of glucose. Without glucose, the animals could not differentiate between the doses of cortisone acetate employed, but when glucose was added, a graded response was obtained.

Fig. 2 also demonstrates that the vehicle in which the cortisone acetate was dissolved influenced the amount of reducing sugar found in the liver. The log dose–response lines illustrated in Fig. 3 indicate that the biological activity of hydrocortisone was greater in oil solution than it was in 10% ethanol.

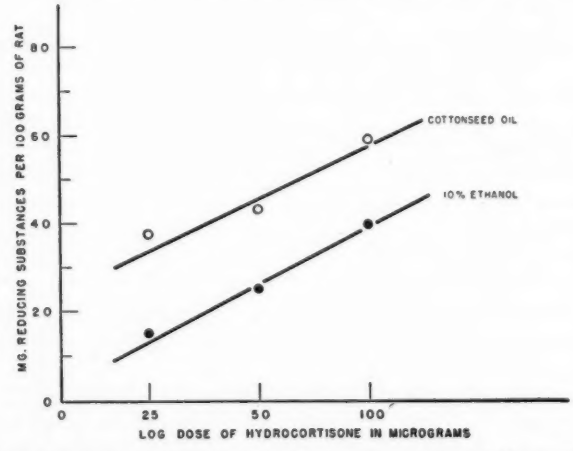

FIG. 3. Log dose–response lines to show the influence of the vehicle on the biological activity of hydrocortisone.

Table V shows the effect of ethanol on the response to cortisone acetate administered in an oil solution. Apparently ethanol depresses the glycogenic response of these animals to adrenal corticoids. It is possible that ethanol influences either (*a*) the mechanism responsible for the deposition of glycogen in the liver, (*b*) the gluconeogenic action of the 11-oxycorticosteroids, or (*c*) the rate of absorption of the hormone from the site of injection.

TABLE V

THE EFFECT OF ETHANOL ON THE RESPONSE OF ADRENALECTOMIZED IMMATURE MALE RATS TO 50 μGM. CORTISONE ACETATE GIVEN IN OIL

No. of rats	Glucose, mgm.	Ethanol 10% solution, ml.	Glucose equivalent per gm. of liver		
			Copper reduction, mgm.	Anthrone reagent, mgm.	Difference, mgm.
8	0	0	4.56 ± 0.46*	2.66 ± 0.56*	1.90
8	100	0	10.84 ± 1.52	10.00 ± 1.72	0.84
8	100	1.0	4.83 ± 0.46	2.88 ± 0.44	1.95

* *Standard error of the mean.*

The Effect of Time After Adrenalectomy on the Precision of the Assay

During the course of this investigation, it was noted that the mortality rate of the adrenalectomized immature male rats increased with the time elapsing between the operation and the test. A series of assays was conducted one day, two days, and three days following adrenalectomy, and it was found that the precision was not adversely affected by performing the test earlier than that reported in other assay procedures of this type (7,14). Table VI gives the average values for the index of precision (s/b) and the coefficient of variation ($s/\bar{y}$) for a number of assays conducted in this study. The differences between the values for s/b are not actually significant although it would appear that tests performed on the day following adrenalectomy

TABLE VI

THE EFFECT OF THE TIME AFTER ADRENALECTOMY ON THE PRECISION OF THE ASSAY

Number of postoperative days	Index of precision, λ	Coefficient of variation, c
1	0.21	27
2	0.33	25
3	0.37	25

provided the best precision. The coefficients of variation support the view that the time following adrenalectomy does not influence the precision of the assays significantly. Consequently, in order to keep the mortality rate at a minimum, the rats were adrenalectomized at 9 A.M., starved overnight, and used in the assay the following day.

Acknowledgment

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MOLECULAR WEIGHT AND HYDRODYNAMIC PROPERTIES OF SODIUM ALGINATE[1]

By W. H. Cook and David B. Smith

Abstract

Sedimentation, diffusion, and viscosity measurements were made on five unfractionated samples of sodium alginate ranging in intrinsic viscosity from 3.1 to 17.5. Diffusion coefficients were subject to large errors and are believed to be overestimated.

Though the molecular weights obtained from sedimentation–diffusion (Svedberg equation) and sedimentation – intrinsic viscosity (Perrin–Simha equations) showed good agreement and yielded values of 3 to 21 $\times$ 10^4, higher values (4.6 to 37 $\times$ 10^4) from sedimentation–viscosity (Mandelkern–Flory equation) appear to be the better estimates. A linear relation between intrinsic viscosity and molecular weight was found with a slope (Mandelkern–Flory equation values) equivalent to $K_m = 13.9 \times 10^{-3}$. The results indicate that sodium alginate has a relatively high extension ratio.

Introduction

Preparations of sodium alginate, an extract from some brown seaweeds, principally from *Laminaria*, usually have high intrinsic viscosities but the source and method of preparation of the extract affect the viscosities greatly. The material is polydisperse, with some evidence of instability in the higher polymers (7). The viscosity of commercially useful preparations indicates a high average molecular weight. As the hydrodynamic behavior of this natural polyelectrolyte is of considerable interest, measurements of intrinsic viscosity and sedimentation and diffusion coefficients made on five samples representing a sixfold viscosity range are reported in this paper.

Sodium alginate is a salt of alginic acid, a linear polymer of D-mannuronic acid residues linked β-1, 4 (4,14). The linear structure has been confirmed by X-ray analysis (2) and, in the solid state, the period along the fiber axis (8.7 Å) indicates a more contracted structure than that of cellulose (10.3 Å).

The following examples reveal some of the discrepancies in reported molecular weight estimates. Heen (13), using viscosity measurements and Staudinger's equation, reported approximate molecular weights ranging from 960 to 15,000. From osmotic pressure measurements, Donnan and Rose (7) obtained number average molecular weights (M_n) ranging from 48,000 to 186,000 for preparations having intrinsic viscosities between 4.2 and 14.4. From sedimentation and diffusion measurements, Säverborn (26) reported weight average molecular weights (M_w) of 50,000 and 75,000 for samples having intrinsic viscosities of 7.7 and 10.3 respectively. These values were not only lower than would be predicted from the results of Donnan and Rose, but in polydisperse systems M_w should exceed M_n. The present work has sought to clarify these discrepancies.

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Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa. Issued as N.R.C. No. 3236.

Experimental

Material

Three laboratory and two commercial preparations of sodium alginate were selected on the basis of solution viscosity. The laboratory samples (AL1, AL2, and AL3), prepared by the methods of Rose (25) and Black (3), were provided by Dr. W. A. P. Black of the Institute of Seaweed Research, Inveresk, Scotland. Sample AL1 was prepared from dried weed and had been stored for some time as dry alginic acid. Samples AL2 and AL3 were prepared from wet weed using every precaution to avoid depolymerization. The commercial samples (AC1 and AC2) were provided by Alginate Industries Limited, Walter House, Bedford Street, London, England. The methods employed for their preparation are unknown but are believed to involve extraction with sodium carbonate and precipitation with calcium chloride.

Chemical Analysis

For analytical purposes, samples were dried *in vacuo* at 45° C. over phosphorus pentoxide to constant weight. Uronic acid analyses by the 19% hydrochloric acid method (20) gave results that increased steadily with time, but the thermal method of Perlin (23) showed a sharp cut-off after 20 min.

TABLE I

SUMMARY OF MEASUREMENTS ON SODIUM ALGINATE

	Sample					
	AL1	AC1	AC2		AL3	AL2
	Solvent					
	Buffer[1]	Buffer	Buffer	1.0 *M* NaCl	Buffer	Buffer
Uronic acid (% of theoretical)	99	96	96	96	97	93
$[\eta]$	(3.1)[2]	5.05 ± 0.08	8.78 ± 0.16	8.15 ± 0.28	16.1 ± 0.29	17.5 ± 0.81
$D_0 \times 10^7$	2.75	1.66	1.35	—	1.16	1.10
$(S^0_{20})_{c\to 0} \times 10^{13}$ ($1/S$ vs. C)	2.08 ± 0.09	2.47 ± 0.06	2.78 ± 0.07	3.03 ± 0.13	3.30 ± 0.21	3.75 ± 0.35
$(S^0_{20})_{c\to 0} \times 10^{13}$ (Eq. 2)	2.08	2.52	2.77	3.03	3.75	4.64
K_s	1.0	1.2	1.9	1.3	3.0	4.8
K'_s	0	0	0	0	− 4.6	−10.6
$(S^0_{20})_{c\to 0} \times 10^{13}$ ($1/S^2$ vs. C)	2.31	2.69	3.27	3.15	4.20	5.15

[1] *Buffer solution 0.15 μ.*
[2] *Based on only four values of reduced viscosity.*

heating. Uronic acid contents obtained by the latter method are given in Table I. Essentially similar values were indicated by ash content and titration procedures. Sample AL2 had the lowest uronic acid content; the composition of the other samples, 96–99% sodium alginate, did not differ significantly from each other and did not indicate a significant content of nonuronide material.

Physical Methods

For physical measurements, samples were dissolved at room temperature in the appropriate buffer or salt solution. These solutions were then dialyzed against 40 volumes of buffer for 40 hr. at 3° C. using cellulose tubing. Tests on the dialyzate with naphthoresorcinol and anthrone showed no polysaccharide in the dialyzate. Unless otherwise stated, the buffer solution consisted of 0.1 *M* sodium chloride plus sodium phosphates to give a pH of 6.6 and a total ionic strength of 0.15. All solutions were saturated with toluene to prevent microbial growth. The concentration of these solutions was determined in a differential refractometer. The refractive index increment, $\Delta n/c$, using yellow light, was 152×10^{-5}/gm./dl.

Intrinsic viscosities were measured in Fenske viscometers at 20°, 25°, and 30° C.

The partial specific volume was determined by the method of intercepts (17) from density measurements made at 25° C.

Diffusion measurements were made on 0.25, 0.5, and 0.75% solutions at 30° C. in a double Claesson diffusion cell using the schlieren scanning apparatus devised by Longsworth (18). The results were corrected to 20° C. by the usual method (1).

Sedimentation rates were measured in a Spinco ultracentrifuge at about 250,000 gravities. The temperature during sedimentation ranged within the limits 25° to 30° C. Results were expressed as S^0_{20} after the usual corrections (1).

Results and Discussion

Partial Specific Volume

The partial specific volume of sodium alginate in the buffer was 0.44 ± 0.01, and that of alginic acid dissolved in 0.045 *N* sodium hydroxide, was 0.59. The latter is in reasonable agreement with Säverborn's figure of 0.605 (solvent 0.2 *N* sodium carbonate) (26) and with values (*ca.* 0.60) reported for neutral polysaccharides. However, with sodium alginate, because of electrostriction and other interactions, the partial specific volume of the mannuronate residue ion could be about 0.5, and the value for the sodium ion may be negative (11). Hence, a value of 0.44 for sodium alginate is reasonable.

Charge Effects

Donnan and Rose (7) and others (12) found that 0.1 *M* sodium chloride was adequate to swamp electroviscous effects. Slightly higher ionic strengths were found necessary to eliminate charge effects in sedimentation. The

S^0_{20} values in solutions of ionic strengths 0.10, 0.15, and 0.20 differed only for the two lowest polymers, being about 10% higher at 0.15 than at 0.10 ionic strength, and about 3.5% higher at 0.20 than at 0.15. The latter difference was applicable only to the lower polymers and was comparable with the experimental error.

Further evidence that charge effects had been neutralized was obtained from sedimentation and viscosity measurements made on sample AC2 in 1.0 *N* sodium chloride. The intrinsic viscosity was only slightly lower and $(S^0_{20})_{c\to 0}$ slightly higher than the corresponding values in the usual buffer (Table I). Moreover, electrophoretic measurements on sodium alginate in 1.0 *N* sodium hydroxide (5) indicated a negligible mobility. Therefore, an ionic strength of 0.15 was considered satisfactory for the physical measurements.

Intrinsic Viscosity

Intrinsic viscosity values are given in Table I. Within the experimental error, intrinsic viscosity was independent of temperature between 20° and 30° C. The results for the most viscous sample, AL2, were considered the least reliable. This sample had the lowest uronide content (Table I), a viscosity falling outside the stability range (7), and was the only sample showing dependence of the viscosity on the rate of shear. The intrinsic viscosity reported for AL2 may therefore be too low.

Diffusion

Diffusion results are included in Table I. Diffusion coefficients (D_m) were obtained from the σ^2 values computed by the usual statistical procedure, from about ten enlarged photographs of the schlieren boundaries taken over 72-hr. diffusion periods. The plot of σ^2 against time showed some scatter about a straight line, but D_m values were reproducible to within about 10% on the lowest polymers, increasing to about 20% for the highest polymer. These errors are comparable with those reported by other investigators (22) on similar material.

The degree of skewness of the diagrams (Fig. 1) indicated greater concentration-dependence of the diffusion coefficient as the polymer size increased. The shape of the diagram for sample AC2 suggested that the commercial material was more polydisperse than laboratory-prepared samples. Diffusion coefficients at infinite dilution (D_0) (Table I) were obtained by an analysis of the patterns and use of the following modification of an equation given by Gralén (10):

$$[1] \qquad D_0 = D_m + \frac{dMo}{d\sigma} \sum_{1}^{n} \frac{A\,\sigma}{4H(t+\Delta t)} \Big/ n$$

This modification was necessitated by the finite value of σ^2 at $t = 0$, which represents the degree of mixing occurring at boundary formation. The addition of a time interval (Δt), equivalent to that required to achieve this degree

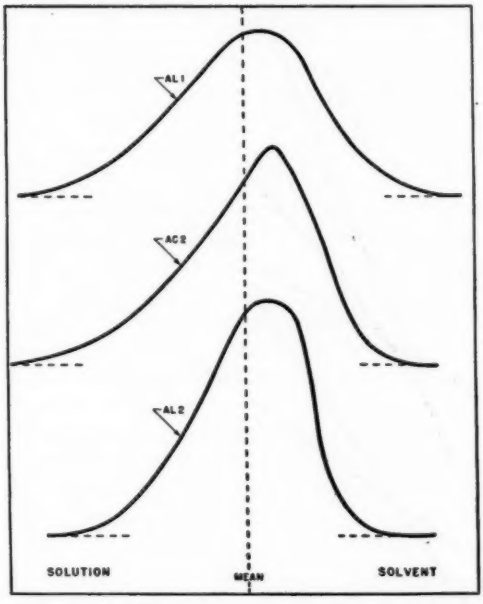

FIG. 1. Representative schlieren diagrams after 50 hr. diffusion of sodium alginate samples.

of mixing by diffusion, was calculated and added to the observed time. However, this simple correction neglected the absence of normal skewing during initial mixing. The absolute degree of skewing was replaced, therefore, with the rate at which skewing (*Mo*, i.e., displacement of the mode from the mean) occurred with σ. This rate and the term within the brackets can both be shown to be constant.

Statistical studies indicated that the D_0 values were subject to errors of about three times the magnitude of those applicable to the D_m values. This enhanced error was partly inherent in extrapolation, but largely arose from the uncertainties of estimating *Mo*, which is small and difficult to measure, but has a marked effect. Low D_0 values subject to large errors operate to produce a systematic bias since these errors appear to be asymmetrically distributed. This bias and the effects of minor vibrations and temperature variations, contribute towards excessively high D_0 values. The accuracy of molecular weight estimates based on the diffusion coefficients of highly concentration-dependent materials is limited to a greater extent than is usually recognized.

Sedimentation

Sedimentation coefficients of all samples were concentration-dependent. Representative results are shown in Fig. 2. The slope of the $1/S$ vs. C plot

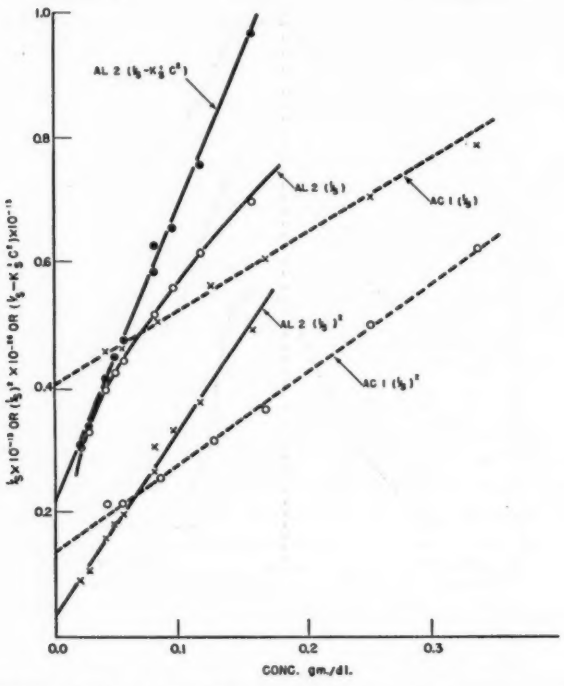

FIG. 2. Representative sedimentation results as a function of concentration.

was not linear for the two most viscous samples, AL2 and AL3, in the lowest concentration range at which measurements could be made, and curvature was evident for all samples at concentrations above 0.4% Sedimentation coefficients at infinite dilution $(S^0_{20})_{c\to 0}$ (Table I) were obtained by linear extrapolation for all samples in the lowest concentration range.

Although a linear relation between $1/S$ and C is frequently found (1), there is no theoretical reason (8) for this behavior. A curved relationship has been observed by Newman *et al.* (22) working with nitrocellulose in ethyl acetate. Their procedure was used for determining the linear (K_s) and second term (K'_s) constants in the equation:—

$$[2] \qquad 1/S^0_{20} = 1/(S^0_{20})_{c\to 0} + K_s C + K'_s C^2 .$$

The values found for these constants are entered in Table I. The K'_s values were zero within experimental error for all samples except AL2 and AL3 (Table I). The $(S^0_{20})_{c\to 0}$ values obtained from Equation 2 were the same as from the linear plot for the lower viscosity samples but 14 and 24% higher for samples AL3 and AL2 respectively. Values obtained from Equation 2 were used in all subsequent calculations.

A plot of $(1/S)^2$ vs. C yielded a straight line for the results on all samples over the full concentration range studied (Fig. 2). Extrapolation of this plot gave $(S^0_{20})_{c\to 0}$ values (Table I) that were about 10% higher for all samples than those obtained from Equation 2. Although this procedure may overestimate $(S^0_{20})_{c\to 0}$, the results are presented to indicate maximum values.

Molecular Weight Estimates

Molecular weight values were calculated using $\bar{V} = 0.44$ on the assumption that the salt was the sedimenting entity. Molecular weights obtained from the sedimentation and diffusion coefficients by the Svedberg equation (29) are plotted against the intrinsic viscosity in Fig. 3. The relation between these quantities was linear except for the most viscous sample, AL2, where the viscosity results are in doubt. The molecular weights lie between 33,000 to 180,000. The method yields weight average values (M_w) for the anhydrous molecule (1). Number average molecular weights (M_n) predicted from the intrinsic viscosities by the Donnan and Rose (7) relation are slightly higher (Fig. 3). The source of the discrepancy, since the M_n value must always be lower than the M_w value with polydisperse material, is believed to lie in the overestimation of the diffusion coefficient.

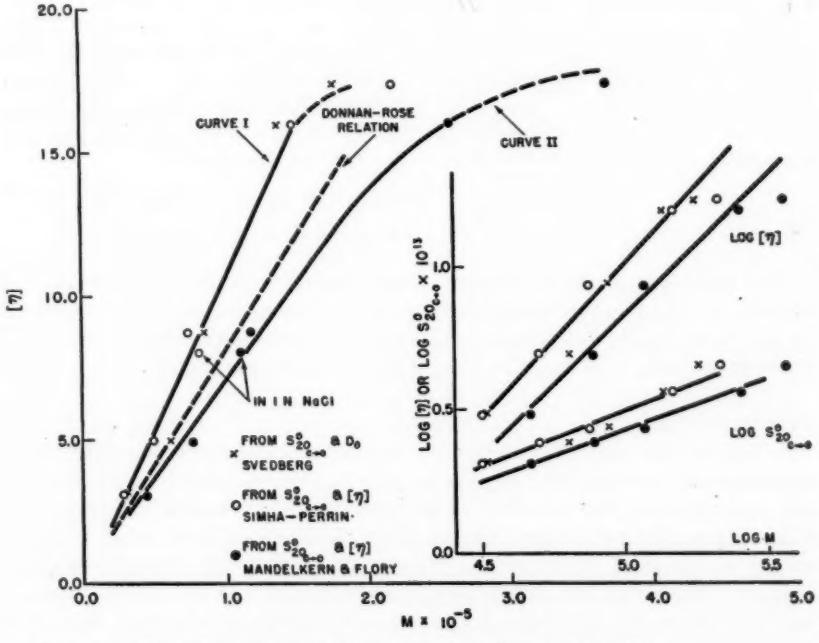

FIG. 3. Relationship between intrinsic viscosity and molecular weight.
Inset. Relationship between log molecular weight and log $[\eta]$ and log $(S^0_{20})_{c\to 0}$.

Molecular weights were also estimated from the intrinsic viscosities and sedimentation coefficients by use of the Simha (28) and Perrin (24) equations, which assume an ellipsoidal anhydrous molecule having an effective volume identical with the partial specific volume, and attribute viscosity enhancement to elongation. The assumptions are of doubtful validity for sodium alginate which would be expected to form a random coil (9). The molecular weights obtained in this way (Fig. 3) are similar, within experimental error, to those obtained from sedimentation and diffusion. In view of the assumptions and approximations used to relate sedimentation and viscosity behavior with molecular weight, this congruence cannot be considered a confirmation of the results obtained by sedimentation–diffusion.

Mandelkern and Flory (19) have recently described a function, $\phi^{\frac{1}{3}}/P$, applicable to linear molecules that form a random coil and from which the molecular weight can be calculated from intrinsic viscosity and sedimentation coefficients. This function is the ratio of the constants of proportionality that relate viscosity and frictional coefficients (equations 3 and 4) to an average linear dimension of the molecule ($\bar{r}$), the root mean square distance between chain ends.

[3] $$[\eta]^{\frac{1}{3}} M^{\frac{1}{3}} = \phi^{\frac{1}{3}} (\bar{r})$$

[4] $$f/\eta_0 = P (\bar{r})$$

While ϕ and P may vary with molecular weight (21), the ratio $\phi^{\frac{1}{3}}/P$ is said to be constant for flexible linear molecules that form a random coil. Experimental evidence and the Kirkwood–Riseman theory (15) give $\phi^{\frac{1}{3}}/P = 2.5 \times 10^6$. Combining Equations 3 and 4, and substituting observed quantities for the frictional coefficient, f, yield the equation:—

[5] $$M^{\frac{1}{3}} = \frac{(S^0_{20})_{c \to 0} [\eta]^{\frac{1}{3}} \eta_0 N}{2.5 \times 10^6 (1\text{-}\bar{V}\rho)}$$

The molecular weights computed from Equation 5 range from 47,000 to 370,000 or 1.4 to 1.8 times higher than those obtained by the other methods. As shown in Fig. 3, they are related linearly, within experimental error, to the intrinsic viscosity except in the case of the most viscous sample.

Cesium Alginate

A test of the assumption that the salt was the sedimenting entity was attempted by converting a sample of sodium alginate (AC2) to the cesium salt and repeating the physical measurements. If the theoretical treatment and experimental values were correct, the molecular weight of cesium alginate should be about 1.55 times that observed for the corresponding sodium alginate. The possibility of altering the size and shape of the molecule during and as a result of conversion limits the conclusions to be drawn from this experiment. A sample of AC2 was converted to the cesium salt by extended dialysis at 3° C. The excess salt was then removed by dialysis against

distilled water at the same temperature, and the sample was freeze-dried and examined in 0.2 *M* cesium chloride. Another portion of sample AC2 was subjected to the same treatment using sodium chloride and was examined in 0.2 *M* sodium chloride.

The sodium alginate control sample showed somewhat higher sedimentation and diffusion coefficients, and a lower intrinsic viscosity than the original AC2, which suggested some change in the polymer during treatment. The cesium salt had a partial specific volume of 0.382. Its $(S^0_{20})_{c\to 0}$ was 4.41 $\times$ 10^{-13}, a value of 1.46 times that of the similarly treated sodium alginate sample, and 1.58 times that of the original AC2. The D_0 value of 1.72 $\times$ 10^{-7} is subject to the same uncertainty as the other diffusion measurements. The intrinsic viscosity of cesium alginate on a weight–volume basis was 3.6 compared with 5.7 for the sodium alginate control sample and 8.8 for the original AC2. Since unit weight of cesium alginate contains less polymer than the sodium salt, a lower viscosity could be expected. When adjusted to the equivalent concentration of sodium alginate, the intrinsic viscosity was 5.6. The molecular weight of the cesium salt from the several methods of computation ranged from 1.2 to 1.7 times that of the control sodium alginate and averaged 1.4 times. However, molecular weight estimates based on uncertain diffusion coefficients or adjusted viscosity values hardly provide the confirmation desired. Since the extended dialysis procedure had some effect on the sodium salt, the average molecular weight increase of 1.4 times was considered to be in reasonable agreement with the expected 1.55. At least the results indicate no gross error in either the measurements or the assumption that the sedimenting and diffusing entity is the undissociated salt.

Molecular Extension

Axial ratios computed (assuming an anhydrous ellipsoidal model) from viscosity (28), sedimentation–diffusion (24), or β values (27), were similar within experimental error. The average values are indicated in Table II for the molecular weights represented in Curve I, Fig. 3. The assumption of an ellipsoidal model, rather than a random coil, is obviously doubtful and axial ratios based on this assumption are doubtless excessive.

Using a random coil model, an estimate of the degree of extension may be obtained by applying the Debye–Bueche (6) theory to the sedimentation results. The pertinent equation is:

$$[6] \qquad f = 6\,\pi\eta_0\,R_s\psi(\sigma) = A\,M^{\epsilon}$$

where f is the frictional coefficient for a single molecule obtained from the molecular weights and sedimentation coefficient, η_0 is the viscosity of the solvent, R_s is the radius of the equivalent sphere, and ψ is a radius factor which is a function of the shielding ratio σ. A and ϵ are constants determined by the experimental results. Values of ψ (σ) and σ corresponding to different values of ϵ have been tabulated by Debye and Bueche.

TABLE II

MOLECULAR EXTENSION ESTIMATES FOR SODIUM ALGINATE

	Sample					
	AL1	AC1	AC2		AL3	AL2
	Solvent					
	Buffer[1]	Buffer	Buffer	1.0 *M* NaCl	Buffer	Buffer
Ellipsoidal model Average axial ratio estimated from $(S^2_{20})_{c\to 0} - D_0$, $[\eta]$, and β values	110	160	210	180	260	260
Random coil $(\bar{r}) = 1.90\ R_s$ Å	320	470	610	610	990	1140
Extension ratio $= \frac{1.4\ (\bar{r})}{\text{D. P.} \times 5.15}$	0.39	0.31	0.29	0.27	0.21	0.17

[1] *Buffer solution 0.15 μ.*

The value of ϵ was determined from the log $(S^0_{20})_{c\to 0}$ vs. log M plots shown as an insert in Fig. 3. The slope of these plots was 0.37 yielding $\epsilon = 0.63$ for both series of molecular weight estimates. The corresponding values of ψ and σ were 0.65 and 3.7 respectively. For small values of σ, the root mean square distance between chain ends $(\bar{r}) = 1.90\ R_s$. The values of $(\bar{r})$ obtained for the several samples are given in Table II. Using the effective length $H = 1.4\ (\bar{r})$ (16) and maximum length (degree of polymerization $\times$ 5.15 $= \frac{5.15 \times \text{mol. wt.}}{198}$), the extension ratios decreased from 0.4 down to 0.2 as the molecular weight increased (Table II). The degree of extension would be somewhat greater if the spacing reported by Astbury (2) for alginate fibers were applicable to dissolved material.

The slope of the log $[\eta]$ vs. log M plot (Fig. 3) yielded $\alpha = 1.0$ for the equation $[\eta] = KM^{\alpha}$. The value of the shielding ratio σ obtained from sedimentation indicated $\alpha = 0.8$. This could mean that the viscosity measurements or the calculated molecular weights were subject to some bias that varied with particle size. The results of Newman *et al.* (22) on nitrocellulose in ethyl acetate, however, reflected a similar behavior. The present work and that of Donnan and Rose (7) indicated that $\alpha = 1.0$ for sodium alginate at $[\eta] < 14$. Harkness and Wassermann (12) using the Donnan–Rose linear relation for estimating M_n from $[\eta]$ reported $\alpha = 0.84$. This value and the molecular extension estimated by these authors seem doubtful. Their data actually indicate extensions comparable with those reported here.

Conclusion

Viscosity, sedimentation, and diffusion measurements yielded similar molecular weights when computed by the Svedberg, or Simha–Perrin equations. Higher values were obtained, however, when the same viscosity and sedimentation coefficients were used in the Mandelkern–Flory equation. In spite of the consistency of the other measurements, the higher value was favored for the following reasons:

(i) The random coil model, on which the Mandelkern–Flory equation is based, should be more applicable to sodium alginate than the ellipsoidal model assumed in the Simha–Perrin equations.

(ii) Although the Svedberg equation should yield molecular weights less dependent on the specific model, the diffusion coefficients were judged to be too high and therefore yielded M_w values that were correspondingly low.

(iii) The M_n values, predicted from the viscosity by the Donnan–Rose relation, yielded values that were comparable with, or even higher than, those obtained from the Svedberg or Simha–Perrin equations. Since sodium alginate is polydisperse, the M_w values, approximated by the measurements used here, should exceed the M_n values. If the Donnan–Rose relation is correct, this condition was only met by the values obtained from the Mandelkern–Flory equation.

Since most investigators (7,12,26) have found a linear relation between intrinsic viscosity and molecular weight within the stability range (i.e., $[\eta] < 15$), the ratio

$$\frac{[\eta]}{\text{Degree of polymerization}} = \frac{198\,[\eta]}{\text{Mol. wt. of Na alginate}} = K_m$$

is a basis for comparison. The values of K_m are listed in Table III. It is evident that the results obtained by Heen (end group titration on low polymers), Donnan and Rose (osmotic pressure), and Curve II, Fig. 3 (sedimentation–viscosity), all yielded similar K_m values (*ca.* 15.0×10^{-3}). Since Heen's value was consistent with that obtained by other methods, his low molecular weights must result from depolymerization in the strong alkali used as solvent. Säverborn's (26) results, either as reported, or when corrected to $\bar{V} = 0.44$, indicate intrinsic viscosities that are too high or, more probably, molecular weights that are too low. Curve I, Fig. 3, also yielded excessively high K_m values and supported the contention that the M_w values obtained from the Svedberg or Simha–Perrin equations were too low.

Application of the Debye–Bueche theory to the sedimentation results indicated a degree of extension that decreased from 0.4 to about 0.2 over the molecular weight range investigated. Even greater extensions were indicated by the frictional coefficient and viscosity measurements using an equivalent ellipsoidal model.

TABLE III

INTRINSIC VISCOSITY – MOLECULAR WEIGHT RELATIONS SODIUM ALGINATE

Source	$K_m = \frac{[\eta]}{\text{D.P.}} \times 10^3$	Remarks
Heen (13)	15.0	Determined by end group titration on low polymers
Säverborn (26)	25.5	Using $\bar{V} = 0.60$
Säverborn	41.9	Average of two points; corrected to $\bar{V} = 0.44$
Säverborn	30.6	Slope between points; corrected to $\bar{V} = 0.44$
Donnan and Rose (7)	15.9	Anhydrous basis
Present investigation	21.5	Svedberg or Simha–Perrin equations
Present investigation	13.9	Mandelkern and Flory equation

Newman and Flory (21) have reported that nitrocellulose esters have a much higher extension ratio at a given molecular weight than is found with most other polymers. Qualitatively, sodium alginate appears to exhibit a similar behavior. While the molecular weights reported here for sodium alginate of a given intrinsic viscosity are somewhat higher than those reported by others, the M_w values are not excessively high. The high viscosity of sodium alginate solutions can therefore be attributed in large part to a high extension ratio.

Acknowledgments

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URINARY EXCRETION PRODUCTS OF MENADIONE (VITAMIN K_3)[1]

By F. C. G. Hoskin, J. W. T. Spinks, and L. B. Jaques

Abstract

Radioactive vitamin K_3 (2-methyl-C^{14}-1,4-naphthoquinone) was administered to rats and the radioactive urinary products separated by paper chromatography and identified by ultraviolet absorption spectra and chemical analyses. Over the dosage range of 2.6 to 11.0 mgm. per kgm. of body weight, 2-methyl-C^{14}-1,4-naphthoquinone was excreted, in order of decreasing relative concentrations as the diglucuronide (Product 1), the monosulphate (Product 2), and a third partially identified derivative of the vitamin (Product 3). Following the administration of vitamin K_3, these products were excreted by normal rats, a dicoumarol-poisoned rat, and a guinea pig. The relative concentrations in the urine of Products 1 and 2 varied directly with the size of dose of vitamin K_3. At these dosages, little or no vitamin K_3 is excreted in urine as such.

Introduction

Relatively few studies have been conducted on the metabolism of vitamin K since its discovery by Dam (6). The speculations of Almquist (2), Quick and Collentine (20), and Lyons (18) that vitamin K may be incorporated into the blood proteins have never been supported by any experimental evidence. In fact Dam, *et al.* (8) found no vitamin K activity associated with the plasma proteins while Solvonuk *et al.* (24), using C^{14}-vitamin K_3, found a little radioactivity attached only to albumin and hemoglobin. An early claim that vitamin K_3 is degraded in the animal organism to phthalic acid which is the active coagulation factor (Shemyakin *et al.* (23)) was shown by Dam (7) and others to be incorrect. A great many publications have described either the identification of glucuronides and ethereal sulphates or the increased urinary excretion of these two following the administration of comparatively large doses of aromatic and aliphatic compounds to animals, and Williams (27) has stated that probably vitamin K_3 is excreted by animals as the glucuronide(s) and/or the sulphate(s). Richert (21) has identified 4-hydroxy-2-methyl-1-naphthyl sulphate in the urine of rabbits which were given orally about 200 mgm. of vitamin K_3 per kgm. of body weight. He also reported an increased glucuronic acid (bound or free) excretion under these conditions. Solvonuk *et al.* (24), following the administration of C^{14}-vitamin K_3 to mice at a dosage level of about 80 mgm. per kgm. of body weight, found a radioactive spot on paper chromatograms of the urine from these animals. When the urine was allowed to stand or was heated with 0.5 *N* acetic acid, two radioactive spots were obtained. Since, as reported here, carbon-14 labelled vitamin K_3 does not give a spot under the chromatographic conditions of Solvonuk *et al.*, an approach is provided to the study of the urinary excretion products of vitamin K_3.

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The present work was undertaken in order to detect and identify the urinary excretion product(s) of vitamin K_3. Dosage levels were selected which, although high compared to dietary requirements, were considerably lower than those previously used, and many times below the toxic level. To this end radioactively labelled vitamin K_3, 2-methyl-C^{14}-1,4-naphthoquinone was administered and chromatographic methods of separation were employed. In addition, because of the small quantities of material encountered, special methods of identification, such as enzymatic hydrolysis, ultraviolet absorption, and isotope dilution, were used.

Materials

Radioactive vitamin K_3.—2-Methyl-C^{14}-1,4-naphthoquinone was synthesized by the procedure of Phillips *et al.* (19). The most highly radioactive vitamin K_3 employed had a specific activity of 1.44 μc. per mgm.

Animals.—Adult male white rats, 230–290 gm. in weight and maintained on the ordinary laboratory chow diet, were used in all except one experiment in which an adult male guinea pig was used.

Methods

Radioactive measurement.—Samples were mounted as infinitely thin layers and counted in a windowless chamber. Vitamin K_3 was counted as the diacetate derivative (Anderson and Newman (3)). The metabolic products of vitamin K_3 were counted as such.

Paper chromatography.—Strips of Whatman No. 1 filter paper, 2 cm. wide, were employed for ascending paper chromatography. The solvent was 3:1 butanol methanol saturated with water. About two drops of glacial acetic acid were added per liter of solvent.

Enzymatic hydrolyses.—β-Glucuronidase (Sigma Chemical Co.) was standardized using phenolphthalein glucuronide and was employed according to the directions of Talalay *et al.* (26). The enzyme was used at a concentration of 10 mgm. of solid preparation per ml. of liquid. Hydrolyses were performed either on filter paper squares impregnated with metabolic products and macerated in the buffer–enzyme system, or on the metabolic products which had been eluted from filter paper squares.

Mylase P was used as a source of arylsulphatase. We are indebted to Wallerstein Laboratories, New York, for a gift of this material. It was standardized using *p*-nitrophenyl sulphate and was employed according to the directions of Huggins and Smith (16). The 45°–50° C. incubation temperature cited as optimum by Abbott (1) was used. The enzyme was employed at a concentration of 10 mgm. of solid Mylase P per ml. of liquid. Hydrolyses were attempted either on filter paper squares impregnated with metabolic products and macerated in the buffer–enzyme system, or on the metabolic products which had been eluted from filter paper squares, or on whole urine containing the metabolic products.

Glucuronic acid analysis.—This was conducted using Tollens' naphthoresorcinol reaction, according to the procedure described by Hanson *et al.* (15), and as modified in this laboratory (Lowenthal and Ogilvie, unpublished). The colored compound formed on heating glucuronic acid (or a glucuronide) with naphthoresorcinol in hydrochloric acid was extracted with *n*-amyl alcohol and optical densities were read at 565 mμ with a Coleman Junior spectrophotometer.

Sulphate estimation.—The method of Johnson and Nishita (17) was tried unsuccessfully. Sulphate ion concentration was therefore estimated turbidimetrically with the aid of a standard series of known concentrations of sodium sulphate. Turbidity was developed by the addition of barium hydroxide to a solution containing sulphate ion (of known or unknown concentration) in an acid medium (approx. 0.1 *N* HCl). That the turbidity (of an unknown) was due to the presence of barium sulphate was confirmed by reducing the suspected barium sulphate to hydrogen sulphide, which was detectable by its odor and the formation of lead sulphide. The reducing mixture and the gas train of Johnson and Nishita (17) were found useful for this.

Ultraviolet absorption spectra.—A Beckman Model DU spectrophotometer was used for the determination of ultraviolet absorption spectra. Because enzyme solutions and filter paper suspensions were extracted, suitable blanks were carried through entire procedures. Both the solutions to be spectrophotometrically examined and their blanks were passed through fine sintered glass filters.

Acid hydrolysis.—The following experiment indicated the conditions probably necessary to hydrolyze any ethereal sulphate derivative of vitamin K_3 (i.e., reduced vitamin K_3, 2-methyl-1,4-naphthohydroquinone) without disrupting the organic moiety.

Solutions, 1×10^{-4} molar with respect to sodium 2-methyl-1,4-naphthohydroquinone disulphate (dihydrate) (Fieser, 13) were made 0.05 *N* to 0.3 *N* with respect to hydrochloric acid and heated in a boiling water bath for one hour. The ultraviolet absorption spectrum of the solution of strongest acidity is shown in Fig. 3 along with that of 2-methyl-1,4-naphthohydroquinone dissolved in water in the presence of air.* Examination of these two spectra indicates that the disulphate derivative of vitamin K_3 was hydrolyzed to the extent of about one hundred per cent but that no further reaction had taken place with the organic moiety. The optical densities at 221 and 251 mμ indicate that greater than ninety per cent of the disulphate derivative had been hydrolyzed even in the 0.05 *N* HCl solutions.

** 2-Methyl-1,4-naphthoquinone and 2-methyl-1,4-naphthohydroquinone dissolved in water in the presence of air gave identical ultraviolet absorption spectra, and were in excellent agreement with that recorded by Davis et al. (9) for 2-methyl-1,4-naphthoquinone.*

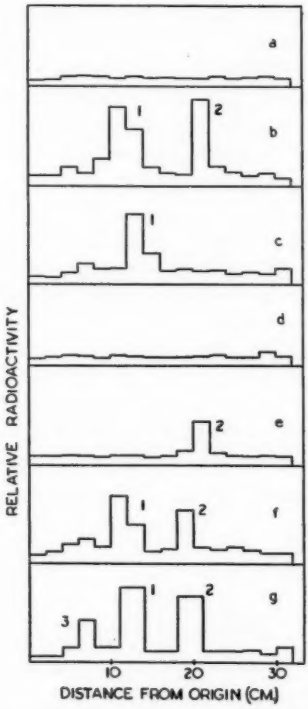

FIG. 1. Histogram representations of paper chromatographs of: (*a*) radioactive vitamin K_3; (*b*) whole urine collected after administration of (*a*); (*c*) a sample of (*b*) after ether extraction; (*d*) Product 1 after incubation with β-glucuronidase; (*e*) Product 2 after incubation with β-glucuronidase; (*f*) a sample of (*b*) after incubation with arylsulphatase (Mylase P); (*g*) similar to (*b*) but at a lower dosage of the vitamin and showing the presence of Product 3.

Experiments and Results

Radioactive vitamin K_3 dissolved in sesame oil was injected intramuscularly into rats at dosage levels of from 3.8 to 11.0 mgm. per kgm. of body weight. The urine was collected for 10 to 20 hr. after the injection during which time 6 to 12 ml. was obtained. From 20 to 40% of the administered radioactivity was excreted in the urine in this volume, and less than ten per cent during the next 20 hr. In all cases at least two radioactive excretion products were detected on the paper chromatograms, as shown in Fig. 1(*b*). One product had an R_f value of about 0.4 (Product 1) and the other about 0.6 (Product 2). By contrast, radioactive vitamin K_3 itself diffused uniformly into the solvent system or evaporated off the paper strips and consequently showed no spot of high radioactivity Fig. 1(*a*). Product 2 (but not Product 1) was found to be extractable from the urine with diethyl ether Fig. 1(*c*).

When the chromatographically separated Product 1 was incubated with β-glucuronidase, it no longer appeared to have a determinable R_f and could

not again be detected on the paper chromatograph Fig. 1(*d*). Product 2, however, was unaltered chromatographically by incubation with β-glucuronidase Fig. 1(*e*). Neither product appeared to inhibit the action of the enzyme on the chromogenic substrate, phenolphthalein glucuronide. Neither Product 1 nor Product 2 was altered by the action of arylsulphatase Fig. 1(*f*), yet neither product appeared to inhibit the action of this latter enzyme on the chromogenic substrate, *p*-nitrophenyl sulphate.

Product 1 was purified twice chromatographically, each time being eluted from its filter paper matrix with cold distilled water. Product 2 was also purified in this manner. The results of glucuronic acid, sulphate, and radioactivity measurements performed on suitable aliquots of these two purified products in solution are shown in Table I.

TABLE I

CHEMICAL COMPOSITION OF PRODUCTS 1 AND 2

	Glucuronic acid (μM.)	Sulphate ion (μM.)	Vitamin K_3 (μM.)
Product 1	2.16 ± 0.26	—	1.00 ± 0.08
	Therefore 2.16 ± 0.31 moles (14.5%) of glucuronic acid per mole of vitamin K_3		
Product 2	—	0.58 ± 0.14	0.49 ± 0.04
	Therefore 1.18 ± 0.30 moles (25.4%) of sulphate per mole of vitamin K_3		

Product 1 was incubated with β-glucuronidase for 20 hr. The buffer–enzyme–substrate system was extracted with ether; the ether was removed; the remaining material was shaken in the dark with distilled water for several hours; and finally this aqueous solution was filtered. Fig. 2 shows the ultraviolet absorption spectra of this solution (i.e., enzymatically hydrolyzed Product 1), of Product 1 before hydrolysis, and of 2-methyl-1,4-napthohydroquinone dissolved in water in the presence of air*. (See previous footnote.)

Product 2 was heated in a boiling water bath with 0.2 *N* HCl for one hour, cooled, and filtered. Fig. 3 shows the ultraviolet absorption spectra of this solution (i.e., acid hydrolyzed Product 2). The ultraviolet absorption spectrum remained unchanged when this solution was made 0.4 *N* in HCl and heated for a second hour. Ultraviolet absorption spectra of Product 2 before hydrolysis, of sodium 2-methyl-1,4-naphthohydroquinone disulphate synthesized by us, and of 2-methyl-1,4-naphthohydroquinone dissolved in water in the presence of air, are shown for comparison. (See previous footnote.)

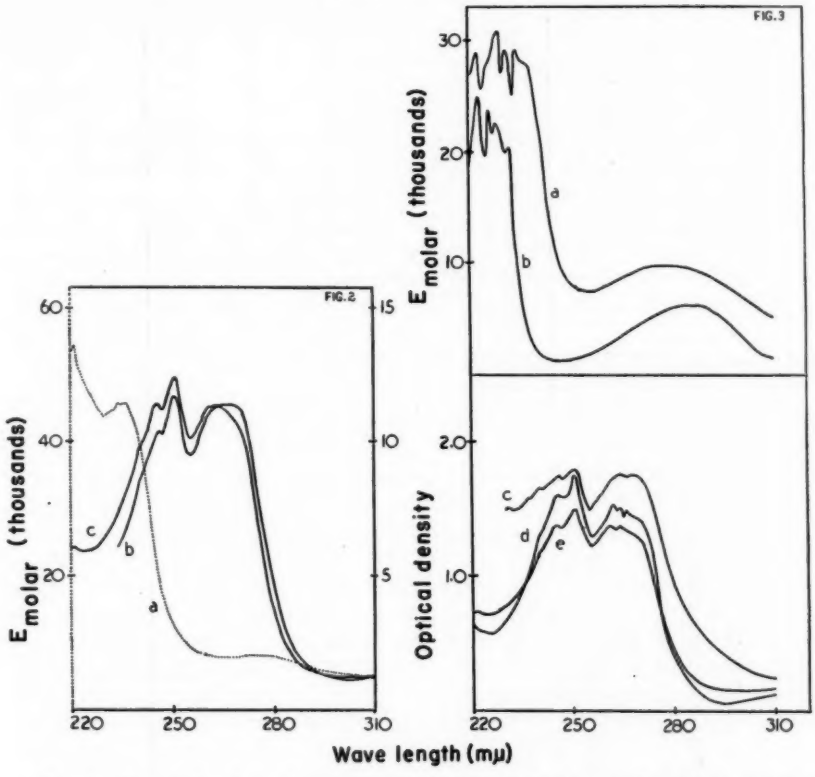

FIG. 2. Ultraviolet absorption spectra of: (*a*-dotted) Product 1; (*b*-solid) the ether extractable portion of Product 1 after enzymatic hydrolysis; (*c*-solid) reduced vitamin K_3 (i.e., the naphthohydroquinone) in solution in the presence of air. (See previous footnote.)

FIG. 3. Ultraviolet absorption spectra of: (*a*) Product 2; (*b*) sodium 2-methyl-1,4-naphthohydroquinone disulphate; (*c*) Product 2 after acid hydrolysis; (*d*) sodium 2-methyl-1,4-naphthoquinone disulphate after acid hydrolysis; (*e*) reduced vitamin K_3 (i.e., the naphthohydroquinone) in solution in the presence of air. (See previous footnote.)

Weighted amounts of nonradioactive vitamin K_3 (Eastman) were shaken with the aqueous solution containing the ether soluble portion of the enzymatically hydrolyzed Product 1 and with the aqueous solution containing the acid hydrolyzed Product 2. Both solutions were extracted with ether and both resulting batches of yellow crystals were recrystallized to give apparently pure vitamin K_3 (m.p. 104°–105° C.). From each batch the diacetate derivative of vitamin K_3 was synthesized and purified to a sharp melting point of 114.5° C. (Anderson and Newman (3)). As shown in Table II, by radioactivity measurement and an application of the isotope dilution principle, more than seventy-five per cent of the radioactivity associated with Product 1 and more than seventy per cent of the radioactivity associated with Product 2

TABLE II

RESULTS OF ISOTOPE DILUTION EXPERIMENTS WITH 2-METHYL-1,4-NAPHTHOQUINONE (VITAMIN K_3)

Subject	Specific activity injected, counts/min./mgm.	"Cold" K_3 added, mgm.[b]	Specific activity of diacetate derivative, counts/min./mgm.	Expected specific activity, counts/min./mgm.	Calculations of expected activity based on
Enzyme-hydrolyzed Product 1	396,000 ± 25,100	26.7	865 ± 53	1105	UV spec. and radioactivity of product
Acid-hydrolyzed Product 2	396,000 ± 25,100	30.4	347 ± 5	486	UV spec. and radioactivity of product
Ether extract of whole urine	1,601,000 ± 40,000	46.5	85 ± 47	22,450	If all injected K_3 excreted as such
				4940	If all excreted radioactivity K_3
Enzyme and acid hydrolyzed[a] Product 1	1,601,000 ± 40,000	30	670 ± 36	810	Estimate of radioactivity from paper chromatograms
Acid-hydrolyzed[a] Product 2	1,601,000 ± 40,000	30	911 ± 24	810	Estimate of radioactivity from paper chromatograms
Acid-hydrolyzed[a] Product 3	1,601,000 ± 40,000	30	215 ± 14	260	Estimate of radioactivity from paper chromatograms

[a] *Performed concurrently.*
[b] *No radioactivity.*

remained with added samples of authentic nonradioactive vitamin K_3 despite repeated purifications and the synthesis of a derivative of vitamin K_3. By contrast, a sample of whole untreated urine collected from a rat which had been injected with radioactive vitamin K_3 was extracted with ether, and the ether was evaporated. The remaining material was taken up in hexane, washed with water, and nonradioactive vitamin K_3 was added to the hexane. By purification of the vitamin K_3, the synthesis of the diacetate derivative, the measurement of its radioactivity and an application of the isotope dilution principle, it was determined that less than three per cent of the radioactivity excreted by the animal and less than one per cent of the radioactivity administered to the animal remained with an added sample of authentic nonradioactive vitamin K_3. The data pertinent to these three isotope dilution experiments are shown in Table II.

In addition to Products 1 and 2, a third radioactive product (R_f about 0.25) was detected. This was first noticed in experiments at the lowest dosage levels of vitamin K_3 in which material of higher specific activity was used (1.44 μc. per mgm.), where it proved to be a significant portion of the radioactivity excreted. This was overlooked at the higher levels of vitamin K_3 having lower specific activity (0.18 μc. per mgm.), as it was then a relatively small percentage of the radioactivity excreted. The presence of this third product is shown chromatographically, in Fig. 1(*g*). Under conditions which effected the enzymatic hydrolysis of Product 1 but not of Product 2 with β-glucuronidase, this third radioactive urinary excretion product (Product 3) appeared to remain unhydrolyzed. The three chromatographically separated products were heated in 0.2 *N* HCl for one hour in a boiling water bath. Isotope dilution experiments similar to those described in the previous paragraph were performed on the solutions resulting from the acid hydrolysis of Products 1, 2, and 3. As shown in Table II(*a*), by radioactivity measurement and an application of the isotope dilution principle, more than eighty per cent of the radioactivity associated with Product 3 remained with an added sample of authentic nonradioactive vitamin K_3 despite repeated purifications and the synthesis of a derivative of vitamin K_3. Table II(*a*) also shows the results of the isotope dilution experiments performed (for a second time) on Products 1 and 2. This third radioactive urinary excretion product was purified by two chromatographic separations and elutions (technique as previously described). The naphthoresorcinol reaction was modified to a spot test (12) and was conducted on samples containing glucuronic acid and on the sample of compound 3 purified chromatographically. In the control sample the concentration of glucuronic acid was adjusted to that calculated for the sample containing compound 3 if all of the radioactivity of the latter were due to a monoglucuronide of K_3. The results of this test showed that glucuronic acid was not present in Product 3. A portion of compound 3 was made 0.3 *N* in HCl and heated to effect hydrolysis. The resulting solution was tested for the presence of sulphate ion using sodium rhodizonate. One test was doubtful, and a second indicated the absence of sulphate ion. Controls of suitable concentration, as well as distilled water, were used.

Table III summarizes the excretion of Products 1, 2, and 3 at various dosage levels of vitamin K_3. The amounts excreted of Products 1 and 2, but not of 3, increased with the dosage of vitamin K_3.

TABLE III

EXCRETION OF PRODUCTS 1, 2, AND 3 AFTER INTRAMUSCULAR INJECTION OF 2-METHYL-C^{14}-1,4-NAPHTHOQUINONE (VITAMIN K_3)

Dosage mgm./kgm.	Animal	μgm. of product per ml. of urine[a]			Vol. voided, ml.	Collection time, hr.
		1	2	3		
2.6	Guinea pig	2	2	14	11	6
3.8	Rat	8	7	5	11	20
4.0	Dicoumarol rat	8	12	4	7	15
6.4	Dicoumarol rat	48	40	5	6	16
6.4	Rat	5	3	1	11	10
7.2	Rat	35	25	6	6	9
11.0	Rat	74	41	6	9[b]	20

[a] *Expressed as μgm. of vitamin K_3.*
[b] *Average value.*

One rat was given 7 mgm. of dicumarol per day orally for six days. Spontaneous hemorrhages were evident from the third day on. This animal was injected with radioactive vitamin K_3 on the third and sixth days, respectively. Paper chromatographs of the urine collected following these injections indicated the presence of the same radioactive products found in previous experiments (without dicumarol) (Table III).

Discussion

Following the administration of vitamin K_3, three excretion products of this compound are found in the urine. The isotope dilution experiment with cold menadione added to the extract of urine after administration C^{14}-K_3 demonstrates that the unconjugated vitamin occurs with these dose levels. One of the urinary products of vitamin K_3 appears to be 2-methyl-1,4-dihydroxynaphthalene-1,4-diglucuronide. The second appears to be a monosulphate derivative of vitamin K_3 and in the light of Richert's (21) observations, it is probably 2-methyl-4-hydroxy-1-naphthyl sulphate. The inability of an arylsulphatase preparation to hydrolyze this latter compound (designated by us as Product 2) may be similar to a case of competitive inhibition reported by Robinson *et al.* (22). The third urinary excretion product of vitamin K_3 (designated by us as Product 3) has been detected and has been partially

identified as a simple, easily hydrolyzable conjugate of vitamin K_3, probably structurally similar to Products 1 and 2. The shift of the major absorption maxima from the region 240–270 mμ for vitamin K_3 to the region 220–240 mμ for the excretion products has also been reported for other conjugated naphthoquinones, i.e., alkylated, acetylated, etc. (Spruit (25), Ewing *et al.* (11)). The unconjugated 2-methyl-1,4-naphthohydroquinone in solution in the presence of air appears to revert readily to the naphthoquinone.

A statistical examination of the data in Table III shows that there is a correlation between the concentration of Products 1 and 2 in the urine and the dosage of vitamin K_3, and between the total amounts of Products 1 and 2 excreted in these experiments and the dosage of vitamin K_3. These correlations are significant at the 5% level, while no significant correlation was found between these values for Product 3 and the dosage of vitamin K_3. Both the concentration and the total amount of Product 3 are low and irregular over the entire range reported here. However, when the concentrations of Products 1 and 2 are calculated *relative to that of Product 3*, a correlation significant at the 1% level or better is found to exist between the relative concentrations of Products 1 and 2 and the dosage of vitamin K_3. A similar relation also exists if the total amounts of the three products excreted in these experiments are used in place of the concentrations.

It is not possible to decide, from these experiments, whether the urinary excretion products detected and identified by us are metabolic products of vitamin K_3 participating in its physiological role, or whether they merely represent detoxication products of 2-methyl-1,4-naphthoquinone, or whether there is any qualitative difference between these two possibilities. The involvement of the liver in the action of vitamin K (Bollman *et al.* (4), Brinkhous (5)) and the relatively high concentrations of arylsulphatase Dodgson *et al.* (10)) and β-glucuronidase (Fishman (14)) in the liver neither clarify nor contradict these two possibilities. It may be pointed out that to date all the work on the metabolism of the K-vitamins has been carried out with K_3 (2-methy-1,4-naphthoquinone) and it should perhaps be emphasized that the results are not necessarily applicable to K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) or K_2 (2-methyl-3-difarnesyl-1,4-napthoquinone).

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PHOSPHORUS METABOLISM OF THE ADRENAL GLAND EFFECT OF HYPOPHYSECTOMY AND ADMINISTRATION OF ACTH ON INCORPORATION OF P^{32} INTO INORGANIC PHOSPHATE[1]

By B. E. Riedel, J. E. Logan, H. A. DeLuca, and R. J. Rossiter

Abstract

In confirmation of the work of others, the concentration of inorganic phosphate (P) in the plasma of hypophysectomized rats was found to be less than that in the plasma of control animals. Hypophysectomy caused no significant change in the concentration of inorganic P in the adrenal gland or liver. A single intraperitoneal injection of each of two preparations of ACTH failed to cause any significant change in the concentrations of inorganic P in plasma, adrenal, or liver.

The specific activity of the inorganic P in the plasma of hypophysectomized rats after an intraperitoneal injection of inorganic P labelled with P^{32} was greater than that in the control animals. Hypophysectomy caused a decrease in the specific activity of the inorganic P of the adrenal gland relative to that of the inorganic P of the plasma. Each of the two preparations of ACTH, given to the hypophysectomized animals as a single intraperitoneal injection 20 hr. before killing, restored the relative specific activity of the inorganic P of the adrenals to normal values. When the ACTH was administered six hours before killing, one of the preparations (ACTH A) caused an increase in the relative specific activity of the inorganic P of the adrenals, but a second preparation (ACTH C) was without significant effect.

The increase in the specific activity of the inorganic P of the plasma comes on slowly (quite small two days after hypophysectomy), whereas the decrease in the relative specific activity of the inorganic P of the adrenal gland comes on rapidly (maximal two days after hypophysectomy). For this reason, at longer time intervals after hypophysectomy (greater than six days) the absolute activity of the acid-soluble P of the adrenal, i.e. the activity not referred to that of the inorganic P of the plasma, was greater in hypophysectomized animals, and not less, as reported by other workers. The activity of this fraction is less in hypophysectomized animals only if the observations are made at short time intervals after removal of the pituitary. Evidence is presented for the view that the increase in the specific activity of the inorganic P of the plasma is the result of changes brought about by a deficiency of growth hormone, whereas the decrease in the relative specific activity of the adrenal is due to a deficiency of ACTH.

Introduction

Collip, Anderson, and Thomson (5) showed that the normal structure and function of the adrenal gland is maintained by an adrenocorticotrophic hormone (ACTH) produced by the anterior lobe of the pituitary. More recently, Gemzell and Samuels (7) described a reduction in the incorporation of radioactive phosphate (P^{32}) into the inorganic phosphate of the adrenals of hypophysectomized rats. There was a decrease in the relative specific activity (i.e. the ratio of the specific activity of the inorganic phosphate (P) of the adrenal to that of the inorganic phosphate of the plasma) 50 min. after an injection of inorganic phosphate labelled with P^{32}. In similar experiments, Reiss and Halkerston (16) reported that the activity of the total acid-soluble

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phosphorus fraction (expressed in counts per mgm. adrenal tissue) was also decreased in hypophysectomized rats. In both instances the incorporation was returned almost to normal by a single injection of ACTH. In addition to these changes in the adrenal, there is also an increase in the specific activity of the inorganic P in the plasma of hypophysectomized rats (7,8).

As a preliminary to a study of the incorporation of P^{32} into other phosphorus-containing compounds (e.g. phospholipids and nucleic acids) of the adrenal gland during conditions of stress, an attempt was made to confirm these observations. When each of two preparations of ACTH was given by a single intraperitoneal injection 20 hr. before the death of the animal, the decrease in the relative specific activity of the inorganic phosphate of the adrenal glands of hypophysectomized rats was abolished. However, in experiments of shorter duration (six hours after the administration of ACTH, instead of 20 hr.), although one of the preparations was found to be active, the other, a preparation of undoubted activity (by ascorbic acid depletion and adrenal weight maintenance tests), was without effect. Presumably, the preparation that was ineffective in these short-term experiments was absorbed more slowly from the site of intraperitoneal injection.

In addition, it has been shown that in hypophysectomized rats the specific activity of the inorganic phosphate of both the plasma and the adrenal depends upon the time that has elapsed since the removal of the pituitary. For this reason, in experiments such as those of Reiss and Halkerston (16) in which the activity of the plasma is not measured, consistent results will be obtained only if the time after hypophysectomy is rigidly standardized.

A preliminary report on some of these experiments has already appeared (18).

Methods

Animals

The animals were all male rats of the Sprague–Dawley strain. Hypophysectomized rats were obtained from the Hormone Assay Laboratories, Inc., Chicago. Macroscopic examination of the pituitary region showed no evidence of pituitary tissue remaining. Microscopic examination of other rats obtained from this source revealed no evidence of pituitary tissue. The adrenal glands showed the typical atrophy associated with hypophysectomy.

The animals were fasted 12–14 hr. before killing. Control animals were carried along with each group of test animals.

ACTH Preparations

Two preparations of ACTH were used. ACTH A was obtained from Armour and Company, Chicago (Batch No. 128-105). It was a buff-colored powder that formed a slightly turbid suspension in isotonic saline. ACTH C was supplied by the Connaught Medical Research Laboratories, Toronto. It was a white powder, completely soluble in isotonic saline. Both preparations were standardized in terms of LA-1-A standard.

Experimental

Test animals received a single intraperitoneal injection of ACTH, 4 mgm. per 100 gm. body weight, in isotonic saline. Control animals received an equivalent volume of isotonic saline. Four hours later all animals received 200 μc. P^{32}*, regardless of body weight, by intraperitoneal injection. The volume of each injection was never more than 0.5 ml.

The animals were killed either 2 or 16 hr. after the administration of the P^{32}. Under nembutal anesthesia, a longitudinal incision was made in the abdomen at the level of the diaphragm. A sample of blood was withdrawn from the inferior vena cava with a No. 18 needle into a 2 ml. syringe containing a small quantity of heparin. The blood was transferred, with care to prevent hemolysis, to a centrifuge tube kept in an ice bath. The blood was subsequently centrifuged in the cold room. The adrenal glands were removed as rapidly as possible, blotted to remove excess blood, and trimmed carefully to remove adhering adipose and connective tissue. They were then plunged into liquid nitrogen and maintained in the frozen state while the liver was removed, trimmed, and frozen in a similar manner.

Analytical and Counting Methods

The two adrenals from each rat were pooled to give a single sample. They were weighed while in the frozen state and homogenized in ice-cold 10% trichloroacetic acid using a homogenizer of the Potter–Elvehjem type. The frozen liver was ground in a mortar and a portion was weighed out and homogenized as described for the adrenals. The specific activity of the acid-soluble inorganic P of the plasma, adrenals, and liver was determined by the method of Ernster, Zetterström, and Lindberg (6). The specific activity of the total acid-soluble P of the adrenal was determined by the same method after ashing with 60% perchloric acid by the method of King (11).

In the method of Ernster *et al.* (6) both the concentration and the radioactivity of the inorganic P are determined on the same sample. The optical density of the blue color complex was first read with a Beckman Model B Spectrophotometer at 730 mμ and the radioactivity due to the P^{32} in the samples was then determined on the same colored solution using an M-6 liquid counter (20th Century Electronics). The counting rate was corrected for background and decay. The specific activities for each animal were then adjusted to a standard injection of 200 μc. per 200 gm. body weight, based on a simulated P^{32} standard (Tracerlab Inc., Boston).

Results

Plasma

Table I shows the effect of hypophysectomy on the concentration and the specific activity of the inorganic P of the plasma two hours after the P^{32} injection. There was a progressive fall in the concentration of inorganic P

* *Supplied in form of* $H_3P^{32}O_4$ *by Eldorado Mining and Refining (1944) Limited.*

TABLE I

PLASMA—INORGANIC P

(Time after ACTH, 6 hr.; time after P^{32}, 2 hr.; mean ± S.E. mean)

Group	No. animals	Body wt. (gm.)	P (mgm./100 ml.)	Spec. activ. (cts./min./μgm. P)	P	
Hypophysectomized 6 days	19	130 ± 0.6	5.42 ± 0.3	826 ± 45	<0.001	
Hypophysectomized 6 days + ACTH A	16	131 ± 1.1	5.02 ± 0.2	700 ± 20		> 0.7
Hypophysectomized 6 days + ACTH C	19	129 ± 1.3	5.23 ± 0.2	806 ± 44		
Hypophysectomized 2 days	30	152 ± 3.4	6.32 ± 0.2	522 ± 17	< 0.01	
Hypophysectomized 2 days + ACTH A	25	153 ± 4.0	6.18 ± 0.2	458 ± 14		> 0.9
Hypophysectomized 2 days + ACTH C	14	148 ± 8.0	6.10 ± 0.2	521 ± 15		
Normal	59	155 ± 2.7	7.58 ± 0.2	464 ± 14	> 0.7	
Normal + ACTH A	22	162 ± 5.3	7.32 ± 0.2	452 ± 17		> 0.4
Normal + ACTH C	22	156 ± 6.2	7.23 ± 0.2	443 ± 18		

with increase in time after removal of the pituitary. Neither preparation of ACTH, when given by a single intraperitoneal injection six hours previously, caused a significant change in the concentration of inorganic P.

In the hypophysectomized animals there was an increase in the specific activity of the inorganic P of the plasma two hours after the administration of the P^{32}. The specific activity was considerably greater at six days than at two days after the operation. The administration of ACTH A caused a small, but statistically significant, decrease in the specific activity of the inorganic P of the plasma in both the six-day and two-day animals. ACTH C was without a significant effect, and neither of the preparations significantly affected the specific activity of the inorganic P of the plasma of normal animals.

In other experiments the effect of hypophysectomy and ACTH on the specific activity of the plasma inorganic P was studied in two-day animals 16 hr. after the administration of the P^{32} and 20 hr. after the administration of the ACTH. No significant differences were found.

Adrenal Gland

Table II shows the effect of hypophysectomy and the administration of ACTH on the concentration of inorganic P in the adrenal and on the incorporation of P^{32} into this fraction 16 hr. after the P^{32} injection. Hypophysectomy caused no significant change in the concentration of inorganic P, nor did the administration of ACTH to either normal or hypophysectomized animals. Table III shows similar results for animals studied six days as well as two days after hypophysectomy.

TABLE II

ADRENAL GLAND—INORGANIC P

(Time after ACTH, 20 hr.; time after P^{32}, 16 hr.; mean ± S.E. mean)

Group	No. animals	P (mgm./100 gm.)	Sp. activ. (cts./min./μgm. P)	Rel. sp. activ. (plasma) × 10^3	P	
Hypophysectomized 2 days	17	23.5 ± 2.6	103	79.1 ± 4.1	< 0.001	<0.01
Hypophysectomized 2 days + ACTH A	18	24.5 ± 2.5	122	106.3 ± 6.3		
Hypophysectomized 2 days + ACTH C	20	22.7 ± 1.6	112	100.4 ± 5.9		
Normal	8	21.2 ± 1.8	121	91.9 ± 7.8	>0.4	>0.3
Normal + ACTH A	8	22.8 ± 2.3	131	99.0 ± 4.3		
Normal + ACTH C	8	22.2 ± 2.0	130	101.3 ± 5.9		

TABLE III

ADRENAL GLAND—INORGANIC P

(Time after ACTH, 6 hr.; time after P^{32}, 2 hr.; mean ± S.E. mean)

Group	No. animals	P (mgm./100 gm.)	Sp. activ. (cts./min./μgm. P)	Rel. sp. activ. (plasma) × 10^3	P	
Hypophysectomized 6 days	19	20.5 ± 0.6	366	44.6 ± 3.1	<0.05	> 0.3
Hypophysectomized 6 days + ACTH A	17	21.3 ± 0.9	374	53.6 ± 3.2		
Hypophysectomized 6 days + ACTH C	20	21.6 ± 0.8	384	48.0 ± 2.2		
Hypophysectomized 2 days	28	20.3 ± 0.8	234	44.8 ± 2.1	<0.001	> 0.5
Hypophysectomized 2 days + ACTH A	20	22.4 ± 0.7	297	65.5 ± 2.8		
Hypophysectomized 2 days + ACTH C	14	18.7 ± 1.0	251	47.9 ± 4.1		
Normal	56	20.4 ± 0.5	347	76.0 ± 3.2	> 0.1	> 0.1
Normal + ACTH A	21	19.4 ± 0.7	312	66.9 ± 3.3		
Normal + ACTH C	22	21.8 ± 0.8	371	86.1 ± 6.0		

In animals studied two days after hypophysectomy there was a decrease in both the specific activity and the relative specific activity of the inorganic P of the adrenal 16 hr. after the P^{32} injection (Table II). The administration of either preparation of ACTH four hours before the P^{32}, i.e. 20 hr. before killing, caused a significant increase in the relative specific activity of the inorganic P of the adrenal in the hypophysectomized animals, but not in the normal controls.

Table III shows similar data for animals studied either two or six days after hypophysectomy. The P^{32} was given two hours previously and the ACTH was given four hours before the P^{32}, i.e. six hours before killing. At two hours after the P^{32} injection the specific activity of the inorganic P of the adrenals of both normal and hypophysectomized animals was considerably greater than at 16 hr., but the relative specific activity was considerably less. As was found in the 16-hr. experiments, both the specific activity and the relative specific activity of the inorganic P in the two-day animals were less than the corresponding figures in the adrenals of the normal controls. However, in the six-day animals the specific activity of the inorganic P of the adrenal had risen to normal, or slightly above. The increase in the adrenal specific activity at six days is the result of the great increase in the specific activity of the inorganic P of the plasma at this time after hypophysectomy (Table I). However, the relative specific activity was significantly decreased at both time intervals after hypophysectomy. The administration of ACTH A caused a significant increase in the relative specific activity at both time intervals after hypophysectomy. After the administration of ACTH C the mean relative specific activity was increased, but the increase was not significant statistically.

Neither preparation of ACTH had a significant effect on the relative specific activity of the inorganic P of the adrenals of normal animals.

Liver

Table IV shows the data for the inorganic P of the liver two hours after the P^{32} injection. Neither hypophysectomy nor the administration of ACTH

TABLE IV

LIVER—INORGANIC P

(Time after ACTH, 6 hr.; time after P^{32}, 2 hr.; mean ± S.E. mean)

Group	No. animals	P (mgm./100 gm.)	Sp. activ. (cts./min./μgm. P)	Rel. sp. activ. (plasma) × 10^2	P	
Hypophysectomized 6 days	7	37.9 ± 4.5	809	114.7 ± 5.6	> 0.3	>0.8
Hypophysectomized 6 days + ACTH A	4	40.0 ± 3.6	805	101.8 ± 9.0		
Hypophysectomized 6 days + ACTH C	6	38.0 ± 5.1	951	112.2 ± 8.8		
Hypophysectomized 2 days	20	36.5 ± 2.2	601	120.4 ± 3.1	> 0.1	<0.05
Hypophysectomized 2 days + ACTH A	14	41.4 ± 2.2	578	126.7 ± 2.7		
Hypophysectomized + ACTH C	6	39.1 ± 0.8	610	133.8 ± 3.3		
Normal	19	38.5 ± 1.5	519	123.4 ± 5.5	> 0.3	>0.05
Normal + ACTH A	4	34.3 ± 1.2	514	135.7 ± 1.3		
Normal + ACTH C	10	33.6 ± 1.8	525	142.0 ± 6.7		

changed the concentration of inorganic P. The specific activities of inorganic P of both the two-day and the six-day animals were greater than the specific activity of the controls, that of the six-day animals being considerably greater. However, these changes were due to the increase in the specific activity of the inorganic P of the plasma (Table I), for the relative specific activity of the liver inorganic P in the hypophysectomized animals was not significantly different from that found in the normal animals for both time intervals after hypophysectomy. Similar data were obtained for the inorganic P of the liver 16 hr. after the P^{32} injection.

From these results it might be supposed that hypophysectomy does not affect the phosphate metabolism of the liver. Such a conclusion would be at variance with the results of Gemzell and Samuels (7), who reported a decrease in the relative specific activity of the inorganic P of the liver 50 min. after the administration of P^{32}. Table IV shows that by two hours after the P^{32} injection the relative specific activity of the inorganic P of the liver exceeded 100 in both normal and hypophysectomized animals. This means that the inorganic P of the liver had reached isotope equilibrium with that of the plasma at some time before two hours, a phenomenon well known for rat liver (9,20). The time at which the equilibrium was attained may have been slightly delayed in the hypophysectomized animals, but the degree of the delay would be very small in comparison with that observed for the adrenal gland. The experiments show that, in general, the time interval of two hours after the P^{32} administration is unsuitable for studying the effect of any factor, such as hypophysectomy, on the relative specific activity of the inorganic P of the liver.

As would be anticipated, neither preparation of ACTH caused a significant change in the relative specific activity of the inorganic P of the liver of either the six-day hypophysectomized or the normal animals. ACTH C, but not ACTH A, caused a slight, and just statistically significant, increase in the two-day hypophysectomized animals.

Discussion

The decrease observed in the concentration of the inorganic P of the plasma of hypophysectomized animals confirms the findings of previous workers (1,7,10,13,21). The failure of a single injection of ACTH to cause an increase is in confirmation of the report of Gemzell and Samuels (7).

The finding that the specific activity of the inorganic P of the plasma of hypophysectomized rats was greater than that in normal animals agrees with the results of Gemzell and Samuels (7) and Geschwind *et al.* (8). The latter workers reported that the increase in specific activity of the plasma of hypophysectomized rats was greatly reduced by the daily administration of growth hormone, an observation which we have confirmed in other experiments not reported in this paper. Since growth hormone also restores the concentration of inorganic P in the plasma of hypophysectomized rats (13), it seems likely that the decrease in the concentration and the increase in the

specific activity of the inorganic P of the plasma brought about by hypophysectomy are the result of changes occasioned by the lack of growth hormone. The increased specific activity is probably due to a combination of several factors, including a decrease in the concentration of inorganic P in the extracellular fluid and a decrease in the volume of extracellular fluid per 100 gm. body weight. We are not aware of any data on the effect of hypophysectomy on the extracellular fluid volume, but the findings of Berlin, van Dyke, Siri, and Williams (4) indicate that there is a considerable decrease in plasma volume. Geschwind *et al.* (8) suggest that the increase in the specific activity of inorganic P of the plasma of the hypophysectomized animals, is possibly an effect secondary to an impaired passage of inorganic P^{32} into the muscle and bone.

Our experiments show that ACTH A caused a significant decrease in the specific activity of the inorganic P of the plasma of hypophysectomized rats, both at two days and at six days after the removal of the pituitary. On the other hand, ACTH C was without effect, and neither preparation was effective in normal animals. The observed difference between the preparations may be due to differences in absorption from the site of injection as commented upon below.

The results for the adrenal confirm the finding of Gemzell and Samuels (7) that in hypophysectomized animals there is a reduced incorporation of P^{32} into the inorganic P fraction. Whether this reduction, as suggested by Gemzell and Samuels (7), is due to a deficiency in the passage of inorganic P^{32} through the membrane of the cells of the adrenal gland is not established by the data presented here, nor by the data presented by Gemzell and Samuels. This point is discussed fully in the following paper (19).

The results with both preparations of ACTH for the 16-hr. experiments and those with ACTH A for the two-hour experiments confirm the findings of Gemzell and Samuels (7) that the decreased relative specific activity of the adrenal inorganic P in hypophysectomized animals is reversed by a single injection of ACTH. The finding that ACTH C produced an effect 16 hr. after the injection of P^{32}, but not at two hours, suggests the possibility that the difference might be due to a delay in the absorption of the ACTH from the site of injection. Other experiments showed that ACTH C was effective, even in short-term experiments, when it was given intravenously. The importance of the route of injection of ACTH and the possibility that much of the evidence for the multiple nature of ACTH might be due to differences in the route of injection and the rate of absorption from the injection site have been commented on by a number of workers (2,3,12,14). These comments become all the more pertinent when the amount of inert material in most ACTH preparations is taken into consideration (2).

Reiss and Halkerston (16) reported a decrease in the incorporation of P^{32} into the total acid-soluble fraction of the adrenals of hypophysectomized animals. These workers expressed their results in terms of counts per 100 mgm. adrenal tissue. This method does not take into consideration the

TABLE V

ADRENAL GLAND—TOTAL ACID-SOLUBLE P

(Time after P^{32}, 2 hr.; mean ± S.E. mean)

Group	No. animals	P (mgm./100 gm.)	Sp. activ. (cts./min./μgm. P)	Activity (cts./min./mgm. tissue)	Rel sp. activ. (plasma) × 10^2
Hypophysectomized 6 days	17	72.0 ± 1.9	155	112	19.4 ± 1.3
Hypophysectomized 2 days	25	71.7 ± 2.5	122	87	20.8 ± 0.7
Normal	51	83.3 ± 1.1	193	161	39.3 ± 2.0

change in the specific activity of the inorganic P of the plasma, which is small at two days, but which is considerable by six days after hypophysectomy (Table I). Table V shows our results for the total acid-soluble P of the adrenal. Two days after hypophysectomy there was a reduction in both the specific activity and in the concentration of the acid-soluble P. These two factors combined to give a significant decrease in the activity (in terms of counts per mgm. tissue) as described by Reiss and Halkerston (16). However, by six days after hypophysectomy, although the concentration of total acid-soluble P remained unaltered, the specific activity had increased considerably, because of the increase in the specific activity of the inorganic P of the plasma (Table I). As a result, the activity, measured in terms of counts per mgm. tissue, had also risen to a value considerably in excess of the two-day figure. On the other hand, the relative specific activity remained the same at six days as it was at two days. In other words, the increase in the specific activity of the plasma, which is due to changes that are probably brought about by a deficiency of growth hormone (8), comes on slowly, being quite small at two days (Table I), whereas the decrease in the relative specific activity of the adrenal, which is due to a deficiency of ACTH, comes on rapidly, being maximal by two days (Table III). These considerations explain the variable, and sometimes puzzling results reported by Reiss, Badrick, and Halkerston (15).

Reiss, Halkerston, Badrick, and Halkerston (17) have suggested the use of the uptake of P^{32} into the acid-soluble P of the adrenal of hypophysectomized rats as a method of assay for ACTH. The results in Table V would indicate that, if the activity of the adrenal (measured in terms of counts per mgm. tissue) is taken, rather than the specific activity relative to that of the plasma, the animals must be used at a standard time interval after the removal of the pituitary. In addition, the differences in the effects observed for the two preparations of ACTH in the two-hour experiments would suggest that either the uptake of P^{32} be measured at a longer time interval after the injection, or that the ACTH be given by the intravenous rather than by the intraperitoneal route.

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PHOSPHORUS METABOLISM OF THE ADRENAL GLAND

THE MECHANISM OF THE CHANGE AFTER HYPOPHYSECTOMY[1]

By B. E. Riedel and R. J. Rossiter

Abstract

The specific activity of the intracellular inorganic phosphate (P) of the adrenal gland relative to that of the inorganic P of the plasma is significantly decreased in hypophysectomized rats at time intervals as long as 16 hr. after the injection of inorganic P labelled with P^{32}. The specific activity of the intracellular inorganic P was determined (1) by measuring the specific activity of the easily-hydrolyzable acid-soluble P, which rapidly comes into isotope equilibrium with the intracellular inorganic P, (2) by calculation, from the specific activity of the inorganic P of the whole adrenal, assuming values for the specific activity and the concentration of the inorganic P of the extracellular fluid and the volume of the extracellular fluid compartment, and (3) by measuring the specific activities of the inorganic P of both the adrenal gland and the plasma at a series of time intervals after the injection of the P^{32}. It is concluded that the decrease in the relative specific activity of the intracellular inorganic P of the adrenal is the result of a slowing in the passage of inorganic P^{32} across the cell membrane, i.e. from the extracellular to the intracellular fluid.

Introduction

Previous workers have shown that hypophysectomy causes a decrease in the incorporation of radioactive phosphorus (P^{32}) into the inorganic phosphate (5) and into the total acid-soluble phosphorus (20) of the adrenal gland of the rat. This decrease was corrected by a single injection of ACTH. These findings were confirmed by Riedel, Logan, DeLuca, and Rossiter (21), although certain differences were observed with different preparations of ACTH.

It is the purpose of the present paper to examine in more detail the nature of the change in the phosphorus metabolism of the adrenal gland. Evidence is presented for the view, suggested by Gemzell and Samuels (5), that in the adrenal gland of hypophysectomized rats there is a slowing in the passage of inorganic P^{32} through the cell membrane.

Hevesy (8,9) has suggested that when inorganic phosphate labelled with P^{32} is administered to an animal, the labelled phosphate rapidly equilibrates with the inorganic P of the extracellular fluid, but that it equilibrates much less rapidly with the inorganic P of the intracellular fluid. In general, because of the unequal distribution of the isotope between the extracellular and the intracellular compartments, the specific activity of the inorganic P of a tissue a short time after the injection of the P^{32} does not give an estimate of the specific activity of the intracellular inorganic P, since the inorganic P of all tissues is partly extracellular (plasma and interstitial fluid) and partly intracellular. Whether the specific activity of the tissue inorganic P will

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give a reasonable estimate of the specific activity of the intracellular inorganic P depends upon the nature of the tissue studied and the time at which measurements are made after the P^{32} injection. Furthermore, a change in the specific activity of the inorganic P of the tissue can be produced by factors other than a change in the true specific activity of the inorganic P of the intracellular fluid, e.g. changes in the relative volumes of the extracellular and intracellular compartments, and changes in the relative concentrations of inorganic P within these compartments.

The adrenal gland is no exception to these general rules. Before one can speculate on the nature of the change in phosphorus metabolism that causes a decrease in the relative specific activity of the inorganic P of the adrenals of hypophysectomized animals, it is necessary, first, to establish whether there is a genuine decrease in the specific activity of the intracellular inorganic P relative to that of the inorganic P of the plasma. Once such a decrease has been demonstrated, it is then permissible to examine its possible causes.

Four methods have been suggested for determining the specific activity of the intracellular inorganic P* of an organ:

1. By measuring the specific activity of the labile P of the adenosine triphosphate (ATP), which rapidly comes into isotope equilibrium with the intracellular inorganic P (9). A rapid incorporation of the isotope into the labile P of ATP has been demonstrated for muscle tissue (2,12), liver tissue (12,14,24), and heart slices (4).

2. By calculation, from the specific activity of the inorganic P of the whole organ, assuming values for (*a*) the specific activity and the concentration of the inorganic P in the extracellular portion of the organ (usually assumed to be that of the plasma) and (*b*) the volume of the extracellular fluid compartment of the organ. This method was used by Hevesy and his associates (10,11) and by Sacks and Altshuler (25).

3. By measuring the specific activity of the inorganic P of the organ and that of the plasma at a series of time intervals after the injection of the P^{32}. At the time interval when these two specific activities are equal, i.e. when the percentage relative specific activity of the inorganic P of the organ is 100, the specific activity of the intracellular inorganic P is equal to that of the extracellular P, which in turn is equal to that of the plasma (24).

4. By removing the extracellular fluid by perfusion, and assuming that the inorganic P that remains is intracellular and has not changed in specific activity during the perfusion (12,26). That these assumptions are not necessarily valid has been pointed out by Ennor and Rosenberg (2).

The first three of these methods have been used in an attempt to determine the specific activity of intracellular inorganic P of the adrenals of normal and hypophysectomized rats.

* *The term intracellular inorganic P is used for the inorganic P that is not in rapid equilibrium with the inorganic P of the extracellular fluid. Perhaps cellular inorganic P would be a better term, for it is possible that much of this inorganic P is concentrated at, or on either side of the membrane.*

Methods

The treatment of the animals, and the analytical and counting methods were described in the previous paper (21). The concentrations and specific activities of the inorganic P of the plasma, adrenal gland, and liver, and of the easily-hydrolyzable acid-soluble P of the adrenal were determined by the method of Ernster, Zetterström, and Lindberg (3). Previous experiments had shown that the rat adrenal contains little phosphocreatine. Most of the labile P is probably derived from ATP.

Results

1. Easily-hydrolyzable Acid-soluble P

Table I shows the concentration and specific activity of the easily-hydrolyzable acid-soluble P of the adrenal of normal and hypophysectomized animals two hours after an injection of inorganic P labelled with P^{32}. Hypophysectomy caused a significant reduction in the concentration of this fraction. The specific activity was decreased two days after the removal of the pituitary, but by six days it had risen again. This rise was the result of the increase in the specific activity of the inorganic P of the plasma and the subsequent increase in the specific activity of the inorganic P of the adrenal (21). However, the specific activity of the easily-hydrolyzable acid-soluble P relative to that of the inorganic P of the plasma was decreased similarly at both time intervals after hypophysectomy.

TABLE I

ADRENAL GLAND—EASILY-HYDROLYZABLE ACID-SOLUBLE P

(Time after P^{32}, 2 hr.; mean ± S.E. mean)

Group	No. animals	P (mgm./100 ml.)	Sp. activ. (cts./min./μgm. P)	Rel. sp. activ. (plasma) × 10^3
Hypophysectomized 6 days	18	13.3 ± 1.0	228	27.5 ± 3.4
Hypophysectomized 2 days	24	11.3 ± 0.7	162	31.0 ± 3.0
Normal	50	18.9 ± 0.8	227	49.2 ± 3.2

If, as has been suggested for other tissue, the easily-hydrolyzable acid-soluble P of the adrenal is in rapid isotope equilibrium with the intracellular inorganic P, these data would indicate that in hypophysectomized animals there is a true decrease in the specific activity of the intracellular inorganic P.

2. Calculation

Table II shows the concentration, specific activity, and relative specific activity of the inorganic P of the extracellular fluid and whole tissue of the adrenal for normal animals and two groups of hypophysectomized animals

whose pituitaries had been removed two and six days previously. The figures for extracellular fluid are those reported for plasma in Table I of the preceding paper by Riedel *et al.* (21) and those for the adrenal are taken from Table II of the same publication. These tables also give the number of animals in each group and the standard errors of the mean values.

TABLE II

ADRENAL GLAND—INORGANIC P

(Time after P^{32}, 2 hr.)

		P (mgm./100 gm.)	Sp. activ. (cts./min./μgm. P)	Rel. sp. activ. (extracell. fl.) × 10^2
Hypophysectomized 6 days	Extracellular fluid	5.4	826	100.0
	Whole tissue	20.5	366	44.4
	Intracellular fluid	24.3	339	**41.0**
Hypophysectomized 2 days	Extracellular fluid	6.3	522	100.0
	Whole tissue	20.3	235	45.0
	Intracellular fluid	23.8	215	**41.3**
Normal	Extracellular fluid	7.6	464	100.0
	Whole tissue	20.4	347	75.0
	Intracellular fluid	23.6	338	**73.0**

From the figures for extracellular fluid and whole tissue reported in Table II, the concentration, specific activity, and relative specific activity of the inorganic P of the intracellular fluid have been calculated. The calculation is based on the assumption that the extracellular fluid compartment comprises 20% of the gland and that the concentration and the specific activity of the inorganic P of the extracellular fluid are the same as those of the plasma. The relative specific activity of the intracellular fluid, in bold type, represents the ratio of the specific activity of the inorganic P in the intracellular fluid to that in the extracellular fluid. Because the specific activity of the tissue inorganic P of the normal animals more nearly approached that of the inorganic P of the extracellular fluid, the values for the specific activity and the relative specific activity of the intracellular fluid were not greatly different from the corresponding values for the whole tissue. For the hypophysectomized animals the relative differences were greater.

In each of the groups of hypophysectomized animals the relative specific activity of the inorganic P of the intracellular fluid was much less than that of the inorganic P of the intracellular fluid of normal animals. If the value of 36%, that obtained by Perlman, Morton, and Chaikoff (17) using the radioactive bromide method, is assumed for the extracellular fluid space of the adrenal, the relative specific activity of the intracellular inorganic P of the hypophysectomized animals would be slightly less than the figure reported in Table II. Also, if the true value for the extracellular fluid space was less

than 20%, or if there was not complete isotope equilibrium between the inorganic P of the interstitial fluid and that of the plasma, the relative specific activity would be greater. However, for the adrenal gland, the correction, unlike that for skeletal muscle at similar time intervals, for extracellular inorganic P is so small that it does not greatly affect the general nature of the conclusions reached.

Table III shows a comparison of the estimates of the decrease in the specific activity of the inorganic P of the intracellular fluid of the adrenal obtained by the two different methods. The first column shows the ratio of the relative specific activity of the easily-hydrolyzable acid-soluble P for the hypophysectomized animals to that for the normal animals. The second column shows a similar ratio for the calculated relative specific activity of the intracellular inorganic P. The agreement between the two estimates of the change in the relative specific activity of the intracellular inorganic P brought about by hypophysectomy is good for both periods after hypophysectomy.

TABLE III

ESTIMATES OF THE CHANGES IN THE RELATIVE SPECIFIC ACTIVITY OF THE INTRACELLULAR FLUID OF THE ADRENAL GLAND

(Ratio (hypophysect./normal) × 100)

——	From easily-hydrol. acid-sol. P	From calc. rel. sp. activ. assuming	
		Extracell. fl. vol. 20% for normal and hypophysect. rats	Extracell. fl. vol. 50% for normal and 10% for hypophysect. rats
Hypophysectomized 6 days	55.9%	56.1%	62.6%
Hypophysectomized 2 days	63.0%	56.6%	63.0%

In the calculations presented in Table II it was assumed that the extracellular fluid volume was the same (20%) in the adrenals of both normal and hypophysectomized animals. A change in the relative volume of the extracellular fluid space of the adrenal, brought about by hypophysectomy, could cause a decrease in the relative specific activity of the tissue inorganic P, with no change in the relative specific activity of the intracellular fluid, only if the extracellular fluid volume were to be relatively less in the adrenals of the hypophysectomized animals. If the extremes of 50% and 10% are assumed for the extracellular fluid volumes of the adrenals of the normal and the hypophysectomized animals, respectively, the calculated ratio of the relative specific activities of the intracellular inorganic P for hypophysectomized and normal rats is still 62.6% for the six-day and 63.0% for the two-day animals (Table III last column). These figures demonstrate conclusively that changes in the relative volume of the extracellular fluid space cannot be responsible for the changes observed in the relative specific activity

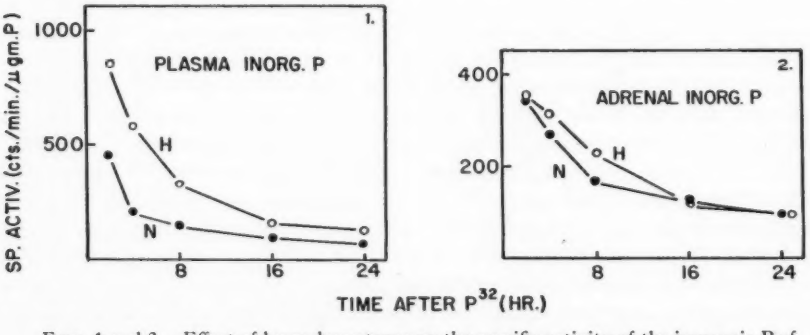
of the tissue inorganic P. Therefore it is probable that the calculated values for the decrease in the specific activity of the inorganic P of the intracellular fluid of the adrenal of hypophysectomized rats are genuine.

3. Measuring Specific Activities at Different Time Intervals After P^{32} Administration

Fig. 1 shows the specific activity of the inorganic P of the plasma at different time intervals after the administration of the P^{32} for normal animals and for animals deprived of their pituitaries 14 days previously. Each point represents the mean for five or more animals and in all instances the differences between the points for normal and hypophysectomized animals are significant statistically ($P < 0.001$). An increase in the specific activity of the plasma inorganic P of hypophysectomized rats has been reported previously (5,6). A similar increase was described for animals studied as early as two days after removal of the pituitary (21).

Fig. 2 shows the specific activity of the inorganic P of the adrenals at different time intervals after the P^{32} injection and Fig. 3 shows similar data for the liver. It can be seen that, despite the great difference in the specific activities of the inorganic P of the plasma (Fig. 1), there was no significant difference between the specific activities of the inorganic P of the adrenals from normal and hypophysectomized animals (Fig. 2). On the other hand, the specific activities of the inorganic P of the liver were much greater in the hypophysectomized animals (Fig. 3), the curve being similar in shape to that reported for plasma (Fig. 1). From these findings it can be deduced that the specific activity of the inorganic P of the adrenal gland relative to that of the inorganic P of the plasma was much less in the hypophysectomized animals (Fig. 4), whereas there was no such difference in the liver (Fig. 5).

Fig. 4 shows that for the adrenal the difference between normal and hypophysectomized animals was greatest two hours after the injection of P^{32} (the time interval for the values reported in Tables I–III), but the difference



FIGS. 1 and 2. Effect of hypophysectomy on the specific activity of the inorganic P of the plasma and the adrenal gland. Time after hypophysectomy, 14 days. H (○–○–○), hypophysectomized rats. N (●–●–●), normal rats. FIG. 1.—plasma; FIG. 2.—adrenal gland.

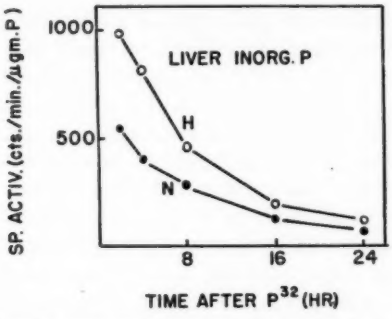
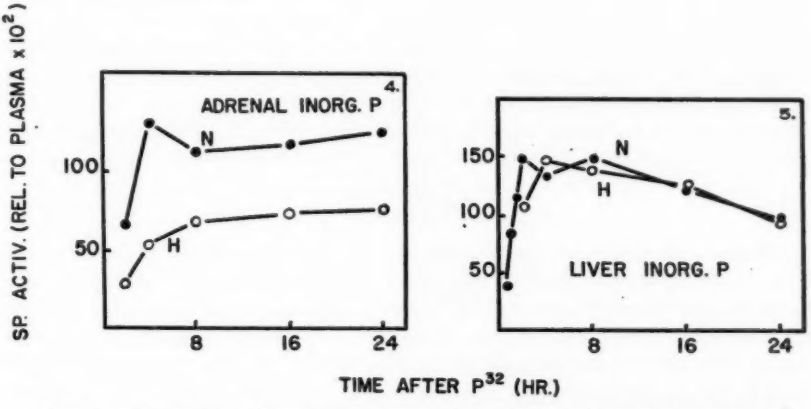


FIG. 3. Effect of hypophysectomy on the specific activity of the inorganic P of the liver. Time after hypophysectomy, 14 days. H (○-○-○), hypophysectomized rats. N (●-●-●), normal rats.



FIGS. 4 and 5. Effect of hypophysectomy on the specific activity of the inorganic P of the adrenal gland and the liver relative to that of the inorganic P of the plasma. Time after hypophysectomy, 14 days. H (○-○-○), hypophysectomized rats. N (●-●-●), normal rats. FIG. 4.—adrenal gland; FIG. 5.—liver.

persisted for 24 hr. By four hours the relative specific activity of the inorganic P of the normal adrenal had exceeded 100. At this time the specific activity of the inorganic P of the gland (which includes both extracellular and intracellular inorganic P) was greater than that of the inorganic P of the plasma.

In hypophysectomized animals the position is quite different. Even after 24 hr., the relative specific activity of the inorganic P of the adrenal did not reach 100. At all periods up to 24 hr. the relative specific activity of the inorganic P of the adrenal of hypophysectomized animals was less than that of the adrenal inorganic P of normal animals. In the adrenals of hypophysectomized animals isotope equilibrium was not attained between the inorganic P of the plasma and that of the intracellular fluid by 24 hr. These results were obtained with animals studied 14 days after the removal of the

pituitary. They confirm the observations made previously with two-day animals that isotope equilibrium was not yet established by 16 hr. after the P^{32} injection (21).

Fig. 5 shows the data for liver. At no time between two and 24 hr. after the administration of the P^{32} were the differences between the figures for normal and hypophysectomized animals significant statistically. These results indicate that in the liver of hypophysectomized animals isotope equilibrium between the inorganic P of the extracellular fluid and the intracellular fluid is established by two hours. The rapid equilibrium of inorganic P^{32} in rat liver is well known (11,24). Because the earliest time interval studied for the hypophysectomized animals was two hours, the data presented here neither confirm nor refute the conclusions of Gemzell and Samuels (5) that the relative specific activity of the inorganic P of the liver of hypophysectomized rats was less than that in the liver of normal rats 50 min. after the injection of P^{32}.

Fig. 4 indicates that for normal animals the time taken for the intracellular inorganic P of the adrenal to come into isotope equilibrium with the inorganic P of the plasma is about three hours. The adrenal is thus intermediate between tissues such as liver and kidney, where the equilibrium is rapid, and tissues such as muscle, testes, and brain, where the equilibrium is slow (9,11).

Discussion

All three methods indicate that the relative specific activity of the inorganic P of the intracellular fluid of the adrenal of hypophysectomized animals is lower than that in the adrenals of normal animals. The most likely reason for a decrease in the relative specific activity of the intracellular inorganic P would be a slowing of the passage of the inorganic P^{32} from the extracellular fluid to the intracellular fluid, i.e. a slowing at 'B'. (See scheme, below.) However, before a slowing at 'B' can be considered as established, other possible causes of the decrease in the relative specific activity of the intracellular inorganic P must be considered. These include (*a*) a slowing at 'A', i.e. a decrease in the rate at which inorganic P^{32} passes from the blood stream to the interstitial fluid and (*b*) an acceleration of one or more of the reactions at 'C'.

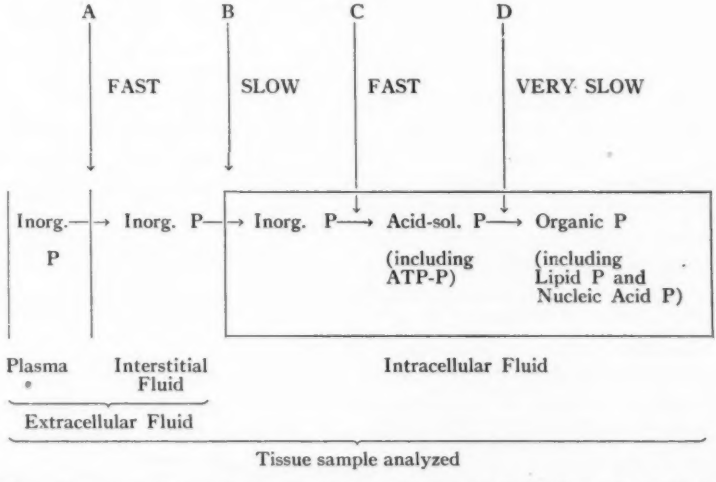
A slowing at 'A', i.e. a decreased rate of passage of inorganic P^{32} from the blood stream into the interstitial fluid of the adrenal would, in all probability, be anatomical in nature, since there is reason to believe that in normal tissues inorganic P^{32} readily penetrates into the interstitial spaces (8,9). An anatomical lesion in a rapidly involuting organ such as the adrenal of the hypophysectomized rat is quite conceivable. However, the rapidity with which recovery takes place, six hours after a single injection of ACTH (5,21), would indicate that the change is the result of a metabolic rather than an anatomical defect. In the early stages, the defect is probably an example of a "biochemical lesion".*

* *The term "biochemical lesion", first introduced by Peters (18) to describe changes that are primarily chemical in nature and usually quickly reversible, has a continued usefulness (19).*

There is little evidence of an acceleration of the reactions at 'C'. This would be characterized by an increase in the specific activity of the total acid-soluble P, and probably also of the easily-hydrolyzable acid-soluble P, relative to that of the intracellular inorganic P. There was no such increase, for Table V of the previous paper (21) and Table I of this paper shows that the decrease in the relative specific activity of these fractions brought about by hypophysectomy was of the same order as the decrease in the relative specific activity of the intracellular inorganic P (Table II).

Another possible cause for a decrease in the specific activity of the intracellular inorganic P in the adrenals of hypophysectomized rats would be an increase in the size of the pool in rapid isotope equilibrium with the inorganic P. This pool would be comprised of either easily-hydrolyzable acid-soluble P or total acid-soluble P. However, Table I of this paper and Table V of the previous paper (21) show that the concentrations of these fractions were less, not greater, after hypophysectomy. In addition, if there were an acceleration of the reactions at 'C' in hypophysectomized animals, one might reasonably anticipate an increase in the specific activity of substances, such as phospholipid, formed by the slow reaction at 'D'. Recently Riedel, Logan, and Rossiter (22) have shown that both the specific activity (relative to that of

SCHEME 1



Schematic representation of the penetration of inorganic P labelled with P^{32} into the phosphorus compounds of the adrenal gland.*

**This representation of the passage of inorganic P^{32} into the cell may be a gross oversimplification. There is some evidence for the view that before the inorganic P^{32} of the extracellular fluid can enter the cell it must first be incorporated into organic P compounds at the cell membrane. These compounds are subsequently broken down into inorganic P (7,13,23,25). There is also evidence that there are diffusion barriers to the free passage of inorganic P within the cell. Crane and Lipmann (1) have shown that in suspensions of washed liver mitochondria added inorganic P exchanges more slowly with the mitochrondrial inorganic P than with the ATP-P.*

the intracellular inorganic P) and the concentration of the lipid P is decreased in the adrenals of hypophysectomized animals.

A further factor to be considered is that, from the time of the removal of the pituitary, the pool of intracellular inorganic P is continually diluted by unlabelled inorganic P derived from the breakdown of lipid P (22), and possibly other P-containing substances, within the cell. Although this breakdown is not sufficiently rapid to alter appreciably the relative specific activity of the intracellular inorganic P at short time intervals after the injection of the P^{32}, it may affect the relative specific activity attained at longer time intervals.

The above considerations make it unlikely that there is either a defect in the passage of the isotope at 'A' or an acceleration at 'C'. The conclusion is that in the adrenals of hypophysectomized animals there is a slowing at 'B', i.e. in the passage of inorganic P^{32} from the extracellular to the intracellular fluid. A single injection of ACTH reverses the change, i.e. causes an increase in the passage of inorganic P^{32} from the extracellular fluid to the intracellular fluid (5,20,21). That the change in the relative specific activity of the inorganic P of the adrenal is associated with the physiological pituitary-adrenal mechanism is shown by the findings of Reiss and Halkerston (20) and Nicholls and Rossiter (15) that there is an increase in the relative specific activity of the intracellular inorganic P of the adrenals in conditions of stress. This effect of stress on the phosphorus metabolism of the adrenal is abolished by hypophysectomy (16,20).

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STUDIES ON THE PRESERVATION OF BLOOD

I. GLYCOLYTIC BEHAVIOR OF BLOOD DURING STORAGE AT 5° C. IN A MEDIUM CONTAINING AN EXCESS OF GLUCOSE[1]

By Hanna M. Pappius[2], Shirley R. Andreae[3], Vernon R. Woodford[4], and Orville F. Denstedt

Abstract

The chemical changes that occur in erythrocytes stored at 5° C. in citrate–glucose medium have been studied. Glycolysis remains normal during the first two weeks of storage. About the 12th to 15th day an impairment in the system occurs at a stage in the glycolytic system before the production of diphosphoglycerate, thus causing a decrease of the concentration of diphosphoglycerate and an accumulation of pyruvic acid. From this time on, the over-all glycolytic activity of the cells is progressively decreased. These indications of failure could not be reversed or retarded by resuspension of the stored erythrocytes in fresh plasma, nor could the onset of failure be made to occur earlier by suspending fresh cells in 'old' plasma.

Introduction

This paper is the first in a series concerning the chemical changes which occur in the erythrocyte during storage under various conditions. The studies represent one aspect of an investigation on the preservation of blood which has been in progress in our laboratory since 1939.

In 1944, Andreae (2), studying the glycolytic behavior of erythrocytes stored in a citrate–glucose medium at 5° C., observed that an abrupt increase in the concentration of pyruvate occurred between the 12th and 15th days of storage. This was found to coincide with an equally abrupt breakdown of diphosphoglycerate as reflected by the liberation of inorganic phosphate. Other workers (2,17,18,19) previously had observed that the inorganic phosphate accumulates in blood during storage and had shown that the rate of hydrolysis of the organic phosphate esters in the erythrocytes could be retarded by the addition of glucose to the specimens. In 1946 Andreae's study in our laboratory was continued by Pappius (20) who subsequently elucidated the significance of the increase in the pyruvate in preserved blood specimens.

Methods

Blood from student donors was collected into a mixture of isotonic trisodium citrate (3.2%) and glucose (5%). The volume ratio of blood to the citrate and glucose solutions in the various experiments was either 5 : 2 : 0.5 or 5 : 1 : 0.5. The metabolic behavior of the red cells during storage at 5° C. was essentially the same in the two mixtures.

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Contribution from Department of Biochemistry, McGill University, Montreal, Que. The material in this paper is taken from theses submitted by H. M. Pappius in partial fulfillment of the requirements for the degree of Master of Science (1948) and of Doctor of Philosophy (1952) in Biochemistry at McGill University.
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The specimens were stored in a refrigerator automatically maintained at 5 ± 1° C. and were agitated gently for a few minutes each day to prevent packing of the sedimented red cells. Samples for chemical analysis were withdrawn aseptically at periods of about three days and were analyzed for glucose (25), lactic acid (14) (modified procedure), pyruvic acid (8), inorganic phosphate (7), and total acid-soluble phosphate (26) by methods indicated by the references.

A fractional analysis of the organic phosphate esters also was carried out in the usual manner (14). The samples were deproteinized with trichloroacetic acid (TCA) and the filtrates hydrolyzed with 1 *N* HCl at 100° C. The inorganic phosphate liberated during the first seven-minute period is derived mainly from the two labile phosphate groups of ATP, and the additional amount, liberated during the remainder of the 100-min. period of hydrolysis, is derived from one of the phosphate ester groups of the hexosediphosphate. The phosphate esters that remain unhydrolyzed after 100 min. include the 2,3-diphosphoglycerate, hexosemonophosphate, which is present in small amount, adenylic acid, and other stable phosphate esters. The amount of the various phosphate fractions was calculated as follows: (*a*) Organic acid-soluble phosphate = total acid-soluble phosphate minus the inorganic phosphate initially present; (*b*) 'hydrolyzable' phosphate = the total inorganic phosphate present after 100 min. hydrolysis minus the amount of inorganic phosphate initially present; (*c*) 'ATP-phosphate' = inorganic phosphate, determined after seven-minute hydrolysis, minus the inorganic phosphate initially present. The amount of 'ATP-phosphate' divided by 2, therefore, represents the concentration of ATP expressed as millimoles per liter. (*d*) The organic phosphate esters which survive the 100 min. boiling in acid comprise the 'stable phosphate' fraction. This quantity is represented by the difference between the total amount of acid-soluble phosphate and the inorganic phosphate at the end of the 100-min. period of hydrolysis. It is advantageous to express the amount of stable phosphate in terms of diphosphoglyceric acid to show more clearly the quantitative contribution of this intermediate to the total quantity of the end-products. Since each molecule of diphosphoglyceric acid yields 2 moles of inorganic phosphate the value obtained for the 'stable' phosphate, expressed as millimoles of phosphate per liter, therefore, should be divided by 2. All the determinations were carried out on duplicate protein-free filtrates. The repeated estimation of total acid-soluble phosphate which should remain unchanged in the preserved specimen, served incidentally as a check on the degree of uniformity of the cell suspension and the constancy of the sampling technique. A hemoglobin estimation was made on each sample as an additional check.

Results

Sequence of Chemical Changes

Figs. 1, 2, and 3 illustrate the typical behavior of glucose, ATP and lactic acid, and diphosphoglyceric acid and pyruvic acid, respectively, in blood

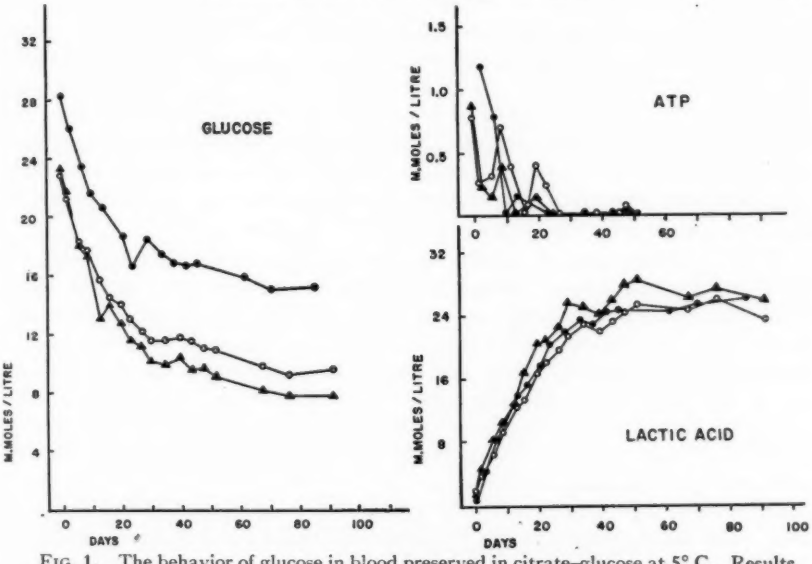

FIG. 1. The behavior of glucose in blood preserved in citrate–glucose at 5° C. Results of three typical experiments. In each case blood was collected into a mixture of isotonic trisodium citrate (3.2%) and glucose (5%). The volume ratio of blood to citrate to glucose was 5 : 2 : 05.

FIG. 2. The behavior of ATP and of lactic acid in blood preserved in citrate–glucose at 5° C. Experimental conditions as for Fig. 1.

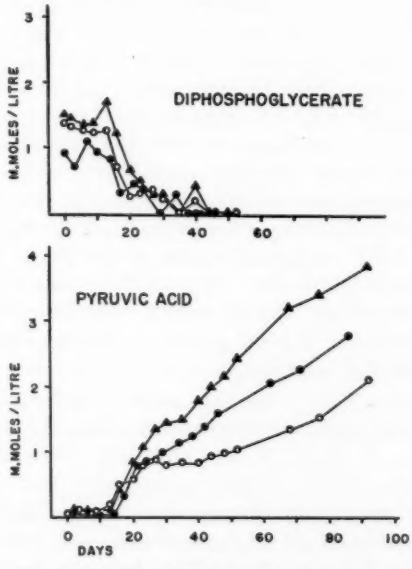

FIG. 3. The behavior of diphosphoglycerate and of pyruvate in blood preserved in citrate–glucose at 5° C. Experimental conditions as for Fig. 1.

preserved in citrate–glucose in three experiments. The same pattern of behavior was consistently obtained in 10 long-term experiments. Table I indicates the changes in the total acid-soluble phosphate, inorganic phosphate, and the 'hydrolyzable' phosphate fractions during storage of the blood at 5° C. The inorganic phosphate values are included in the table, but since the increase in this fraction originates from the breakdown of the organic phosphate esters it is necessary to discuss only the behavior of the diphosphoglycerate, 'hydrolyzable' phosphate, and ATP fractions.

It may be seen from the above figures that the changes in the glycolytic behavior of blood during storage in citrate–glucose medium occur in three main stages. The first stage extends over the first two weeks of storage, during which period the glucose content of blood specimen undergoes a steady decrease. Concurrently, lactic acid accumulates although, up to this time, not all of the glucose can be accounted for in terms of lactic acid. Until the 12th–15th day, the concentration of pyruvate and diphosphoglycerate undergoes little, if any, change. Thus, during the first stage the glycolytic activity of the red cells proceeds in a normal manner with the disappearance of the primary substrate—glucose, and the accumulation of the end-product—lactic acid. The concentration of the glycolytic intermediates remains unchanged, that is, a balance is maintained between the rate of their formation and removal.

The second phase, which usually occurs during the third and fourth weeks of storage, is characterized mainly by a decrease in the concentration of the diphosphoglycerate and an accumulation of pyruvate. During this period the rate of breakdown of glucose and the rate of lactic acid formation undergo a gradual decrease, and thus, part of the glycolytic system appears to be diminished in activity. The changes observed in the concentration of diphosphoglycerate and pyruvate suggest that the rate of formation of diphosphoglycerate does not keep up with the rate of its conversion into pyruvate. The latter product tends to accumulate indicating that its formation from diphosphoglycerate is unimpaired, but its conversion to lactate is progressively diminished, presumably owing to the steady decrease in the amount of reduced DPN as a consequence of the diminution of the rate of formation of diphosphoglycerate. Apparently, therefore, the progressive impairment of the glycolytic activity of the cells during the second stage is the result of an impairment of some part of the mechanism above diphosphoglycerate in the glycolytic sequence.

The concentration of ATP in the cells tends to fall steadily during storage at 5° C. and usually becomes depleted by the 30th day in blood stored with added glucose.

It may be seen from Table I that the 'hydrolyzable' phosphate fraction, representing mainly the two labile phosphate groups of ATP and one from hexosediphosphate, is increased during the first week of storage of blood in the citrate–glucose medium at 5° C. This increase invariably occurs, and indicates an increase in the concentration of hexosediphosphate since the

TABLE I

THE BEHAVIOR OF TOTAL ACID-SOLUBLE PHOSPHATE, THE INORGANIC PHOSPHATE, AND THE "HYDROLYZABLE" PHOSPHATE IN BLOOD PRESERVED IN CITRATE–GLUCOSE AT 5° C.

Duration of storage, days	Total acid-soluble phosphate	Inorganic phosphate	"Hydrolyzable" phosphate
	Millimoles phosphate per liter of whole blood		
0	5.00	1.32	0.89
2	5.00	1.05	1.27
5	5.10	1.37	1.21
9	4.89	1.21	1.21
13	5.10	2.63	1.21
16	5.05	3.47	0.16
20	4.89	3.52	0.79
23	5.05	4.31	0.11
27	5.36	5.05	0.00
30	5.15	4.89	0.00
35	5.21	5.11	—
40	5.32	5.11	—

NOTE: *The blood was collected into a mixture of isotonic trisodium citrate (3.2%) and glucose (5%). The volume ratio of blood, citrate, and glucose was 5 : 2 : 0.5.*

concentration of ATP is decreased during the same period. During the second week of storage the amount of hydrolyzable phosphate tends to undergo a further steady decrease and usually is depleted about the fourth week.

The third phase—from about the 30th day of storage onward—is characterized by a marked retardation in the rate of utilization of glucose and a correspondingly retarded rate of formation of lactate and pyruvate. Blood specimens from various donors, when preserved in citrate–glucose medium, tend to show considerable difference in behavior with respect to the accumulation of pyruvate during this period. It may be seen from Fig. 3 that the total amount of pyruvate in different samples varied between 1.5 mM. and 3.0 mM. During the third phase, the concentration of diphosphoglycerate approaches zero. This period of storage, therefore, is characterized by a pronounced deterioration of the glycolytic system.

The Influence of the Accumulation of End-products on the Glycolytic Activity of Blood During Storage

It was important to ascertain whether the accumulation of lactate or the depletion of some diffusible factor during storage is in any way responsible for the decrease in the glycolytic activity and particularly for the characteristic changes which occur about the 14th day of storage in blood preserved in citrate–glucose at 5° C.

A sample of blood, collected into the isotonic citrate–glucose diluent (blood: citrate: glucose, 5 : 2 : 0.5), was stored at 5° C. On the 7th, 14th, and 21st day, respectively, of storage, an aliquot was removed, the red cells were sedimented by light centrifugation and, after separation from the supernatant

TABLE II

THE INFLUENCE OF SUSPENDING PRESERVED ERYTHROCYTES IN FRESH PLASMA AND OF SUSPENDING FRESH ERYTHROCYTES IN AGED PLASMA ON THE GLUCOSE UTILIZATION BY THE CELLS AT 5° C.

Duration of storage, days	Sample					
	A	B	C	D	E	F
	Millimoles glucose per liter of whole blood					
0	19.10	18.66	18.21	20.22	17.33	18.89
2	—	—	—	—	—	17.55
3	—	—	—	—	15.78	—
5	17.55	—	—	18.44	—	16.72
6	—	17.33	—	—	14.44	—
7	16.11	—	16.00	—	—	—
—	17.55	—	—	—	—	—
8	—	—	—	—	—	15.50
9	—	—	—	—	14.83	—
10	16.11	—	—	16.11	—	—
12	—	15.33	—	—	—	14.33
13	15.33	—	14.89	15.78	—	—
14	—	14.78	—	—	13.16	—
—	—	19.89	—	—	—	—
15	—	—	—	—	—	12.77
16	15.39	20.11	—	15.78	—	—
17	—	—	—	—	13.06	—
19	14.56	20.05	14.72	15.88	—	12.44
21	—	—	14.11	—	12.16	—
—	—	—	17.33	—	—	—
22	14.06	19.40	—	15.16	—	—
23	—	—	—	—	—	11.44
24	—	—	16.89	—	—	—
26	13.83	18.60	—	14.55	11.88	—
27	—	—	16.33	—	—	—
29	—	—	—	13.44	—	—
30	12.77	17.83	16.50	—	11.44	—
33	—	—	16.22	13.66	—	—
34	12.88	17.11	—	—	10.61	—
37	—	—	15.11	13.22	10.61	—
41	—	16.44	14.77	12.66	—	—
44	11.72	—	—	—	—	—

NOTE: *In all samples blood was collected into a mixture of isotonic trisodium citrate (3.2%) and glucose (5%). The volume ratio of blood, citrate, and glucose was 5 : 2 : 0.5.*

A—Cells resuspended in fresh plasma after seven days' storage at 5° C.

B—Cells resuspended in fresh plasma after 14 days' storage at 5° C.

C—Cells resuspended in fresh plasma after 21 days' storage at 5° C.

D—Control—sample stored untreated.

E—Fresh cells resuspended in plasma which was removed from a specimen which had been stored for seven days.

F—Fresh cells resuspended in plasma which was removed from a specimen which had been stored for 14 days.

plasma, were resuspended in fresh plasma obtained from the same donor. The red cells from the freshly taken blood samples were suspended in the plasma that had been removed from the stored specimen. Care was taken to ensure a uniform concentration of red cells in the various samples.

It may be seen from Table II that suspension of the preserved red cells in fresh plasma (samples A, B, and C) did not alter the rate of utilization of glucose from that of the control (sample D). Similarly with samples E and F in which the freshly-drawn cells had been suspended in 'old' plasma, the rate of glycolysis was the same as in the control specimen.

TABLE III

THE INFLUENCE OF SUSPENDING PRESERVED ERYTHROCYTES IN FRESH PLASMA AND OF SUSPENDING FRESH ERYTHROCYTES IN AGED PLASMA ON THE ACCUMULATION OF LACTIC ACID AT 5° C.

Duration of storage, days	Sample*					
	A	B	C	D	E	F
	Millimoles lactic acid per liter of whole blood					
0	1.50	1.16	1.55	1.83	5.05	7.77
2	—	—	—	—	—	9.30
3	—	—	—	—	7.27	—
5	5.83	—	—	5.94	—	13.61
6	—	6.11	—	—	9.72	—
7	7.38	—	7.50	—	—	—
—	4.33	—	—	—	—	—
8	—	—	—	—	—	15.41
9	—	—	—	—	10.00	—
10	6.55	—	—	9.11	—	—
12	—	10.83	—	—	—	16.80
13	7.91	—	—	11.80	—	—
14	—	10.83	—	—	14.16	—
—	—	5.41	—	—	—	—
15	—	—	—	—	—	19.16
16	9.30	5.69	—	11.27	—	—
17	—	—	—	—	15.83	—
19	11.53	8.19	14.72	14.03	—	23.19
21	—	—	15.00	—	17.92	—
—	—	—	6.80	—	—	—
22	12.22	9.44	—	14.16	—	—
23	—	—	—	—	—	24.03
24	—	—	8.47	—	—	—
26	13.33	11.11	—	15.27	18.61	—
27	—	—	9.30	—	—	—
29	—	—	—	17.08	—	—
30	13.47	11.39	8.19	—	18.61	—
33	—	—	9.86	18.33	—	—
34	16.25	14.03	—	—	18.88	—
37	—	—	10.83	17.77	20.13	—
41	—	14.53	11.25	18.33	—	—
44	16.94	—	—	—	—	—

* *See footnote, Table II.*

A similar parallelism in the behavior of lactic acid in all the samples is evident from Table III. The rate of accumulation of lactate was comparable in the various specimens whether the initial concentration of lactate was relatively low following suspension of cells in plasma, as in samples A, B, and C, or was above normal at the beginning of storage as in samples E and F.

The time when pyruvate began to be accumulated and the rate of accumulation (cf. Table IV) were not altered by suspension of the cells in fresh plasma on the seventh day of storage (sample A). Nor did suspension of the preserved

TABLE IV

THE INFLUENCE OF SUSPENDING PRESERVED ERYTHROCYTES IN FRESH PLASMA AND OF SUSPENDING FRESH ERYTHROCYTES IN AGED PLASMA ON THE ACCUMULATION OF PYRUVIC ACID AT 5° C.

Duration of storage, days	Sample*					
	A	B	C	D	E	F
	Millimoles pyruvic acid per liter of whole blood					
0	0.034	0.045	0.040	0.028	0.023	0.114
2	—	—	—	—	—	0.057
3	—	—	—	—	0.040	—
5	0.034	—	—	0.034	—	0.040
6	—	0.034	—	—	0.028	—
7	0.017	—	0.028	—	—	—
—	0.023	—	—	—	—	—
8	—	—	—	—	—	0.040
9	—	—	—	—	0.028	—
10	0.023	—	—	0.125	—	—
12	—	0.148	—	—	—	0.216
13	0.210	—	0.051	0.460	—	—
14	—	0.335	—	—	0.455	—
—	—	0.125	—	—	—	—
15	—	—	—	—	—	0.494
16	0.489	0.227	—	0.625	—	—
17	—	—	—	—	0.665	—
19	0.597	0.295	0.369	0.653	—	0.739
21	—	—	0.210	—	0.796	—
—	—	—	0.062	—	—	—
22	0.710	0.273	—	0.738	—	—
23	—	—	—	—	—	0.881
24	—	—	0.102	—	—	—
26	0.738	0.233	—	0.682	0.852	—
27	—	—	0.153	—	—	—
29	—	—	—	0.653	—	—
30	0.739	0.176	0.199	—	0.881	—
33	—	—	0.256	0.653	—	—
34	0.795	0.199	—	—	0.966	—
37	—	—	0.369	0.653	0.966	—
41	—	0.244	0.369	0.739	—	—
44	1.051	—	—	—	—	—

* *See footnote, Table II.*

cells in fresh plasma, after pyruvate had begun to accumulate, alter the rate of accumulation (samples B and C). In the latter two cases the final concentration of pyruvate was lower than in the control sample D, owing to the removal of the pyruvate along with the plasma.

It may be observed that the abrupt rise in the pyruvate occurred coincidentally in samples D, E, and F. It is evident, therefore, that there was no difference in the time when this change took place whether the cells were suspended and stored in fresh plasma or in 'old' plasma taken from blood samples which had been stored for several days. Although the rate of accumulation of pyruvate during subsequent storage appeared to be somewhat greater in the specimens with the 'old' plasma the differences were within the normal limits of variation in blood specimens taken from different individuals (cf. Fig. 3).

Discussion

It is well known that the glycolytic system in blood is confined to the formed elements (12,16), although this point was disputed by some of the earlier workers (11). The relative contribution of the erythrocytes and the leucocytes to the total glycolytic activity of the blood is about 8 : 1 (4,15). Since the numerical ratio of the two types of cell in the blood is approximately 1000 : 1, it is apparent that the white cells have by far the greater glycolytic activity. However, it is known that the white cells in cold-preserved blood specimens disintegrate within a few days (5), and thus the continued glycolytic activity in such specimens is attributable almost exclusively to the red cells. This was established by Andreae (2) who studied the glycolytic activity of preserved samples and obtained comparable results with whole blood and red cell specimens which had been washed in the cold to remove the white cells and the plasma.

The spontaneous hemolytic breakdown of the red cells in blood preserved in citrate–glucose at 5° C. begins and proceeds slowly after four to six weeks of storage, by which time the glycolytic activity of the specimen has become greatly diminished. Furthermore, as will be shown in a later paper in this series, the glycolytic activity of samples containing citrate is confined to the intact cells.

The role of the 2,3-diphosphoglycerate in the glycolytic system of the erythrocyte is of great interest. This stable compound, which is present in other somatic cells only in traces, is not generally regarded as being an intermediate in the Meyerhof Cycle, but merely as playing a catalytic role in the conversion of 3-phosphoglycerate to 2-phosphoglycerate (27). However, in most mammalian species it is present in the erythrocytes (10,22), and it makes up about 50% of the organic phosphate compounds in the human red blood cell. Rapoport and Luebering (23,24) have obtained evidence of the existence, in rabbit erythrocytes, of a mutase, which catalyzes the transfer of phosphate from the 1,3- to the 2,3-diphosphoglycerate, without the intermediation of a nucleotide. These authors point out that the reaction differs

from that catalyzed by the other mutases by being accompanied by a fall in free energy amounting to more than 10,000 calories under standard conditions. This fall in energy is associated with the transfer of phosphorus from the carboxyl to the secondary alcohol group of the glyceric acid. The reaction, like that catalyzed by the hexokinase, is irreversible. It would appear therefore that the 2,3-diphosphoglycerate must be dephosphorylated directly to the 3-monophosphoglyceric acid and that its further breakdown does not proceed by way of the 1,3-diphosphoglycerate.

Recently, Gourley (9) obtained confirmatory evidence indicating that 2,3-diphosphoglycerate is connected with the glycolytic cycle of human erythrocytes in the manner postulated by Rapoport and Luebering (23,24). The close relationship between the behavior of diphosphoglycerate and pyruvate observed in our studies indicates that the former is at least in equilibrium with the glycolytic system in the erythrocyte.

The behavior of glucose, lactic acid, and the various phosphate fractions in blood stored at 5° C. in the citrate–glucose media described here is in general agreement with the observations of other workers in this field (3,17–19,21). However, our studies on the behavior of pyruvic acid have thrown additional light on the mechanics of the failure of the glycolytic mechanism in blood during storage.

Our interpretation of the results is as follows: Glycolysis proceeds in a normal manner in blood preserved in citrate–glucose medium at 5° C. during the first two weeks of storage. About the third week of storage the activity of one or more reactions in the glycolytic series between the breakdown of glucose and the production of diphosphoglycerate becomes impaired and, as a consequence, the rate of formation of diphosphoglycerate does not keep up with the rate of its further metabolism. With the diminished formation of diphosphoglycerate the rate of reduction of DPN is decreased, and since the reduced DPN is required for the reduction of pyruvate to lactate, the latter process also becomes impaired. Thus pyruvate tends to accumulate since the rate of its formation exceeds that of its removal. The decreased rate of utilization of glucose and of production of lactic acid during the second phase of storage thus may be explained and the accelerated rate of failure during the third phase is to be expected.

The factor or condition which initiates the failure of glycolysis in blood during storage, particularly when ample glucose is present in the medium, still has not been established, nor is it known exactly where in the glycolytic system the impairment first occurs. It is evident from the experiment on the resuspension of stored cells in fresh plasma and of fresh cells in 'old' plasma, that the increase in the lactic acid concentration as a result of the glycolysis of glucose or the accumulation of other products, is not the initiating factor since the glycolytic activity of the cells was not affected by the plasma. The diminished utilization of glucose after the second week of storage has been attributed to decreased concentration of ATP (21). The same sequence of change, however, would result equally from a fall in the concentration of

DPN or from a diminution in the activity of the coenzyme. We have evidence which indicates that DPN is broken down at a slow rate during storage. However, at present we are not certain as to which of the two coenzymes is the more closely involved in the initiation of the gradual failure of glycolysis. It is unlikely that the failure of certain of the enzymes is due to alteration of the apoenzyme component since apoenzymes in general are known to remain unaltered during long periods of storage (1,6,13).

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CARDIOVASCULAR RESPONSES IN DOGS TO LARGE INTRAVENOUS INFUSIONS[1,2]

By C. W. Gowdey, J. D. Hatcher[3], and F. A. Sunahara

Abstract

The effects of a continuous, slow, intravenous infusion of gum acacia solution have been measured in anesthetized dogs. When the volume of the circulation was increased and the hematocrit value reduced, the cardiac output, intracardiac pressures, and peripheral blood flows began to increase. In some experiments these changes continued until sudden cardiac decompensation occurred during which the arterial blood pressure, cardiac output, and peripheral blood flows were reduced while the mean right auricular and ventricular pressures increased markedly. Analysis of the results indicates that up to the time of the highest cardiac output there is a closer correlation between cardiac output and hematocrit value than between cardiac output and mean right auricular pressure.

Introduction

Earlier work in hypervolemia was concerned with relatively few of the physiological variables, and most of it was done with isotonic saline or glucose solutions. More recent work with macromolecular plasma substitutes has pertained mainly to their excretion rates and possible toxicity. Wiggers and Katz (31) found that rapid saline infusions in dogs caused an increased right atrial pressure which, above a critical level, was associated with cardiac decompensation. Others (30,33) reported that massive infusions of saline or glucose produced some of the phenomena of congestive heart failure. Meek and Eyster (19) found no increase, or only transient increases, of the diastolic size and output of the heart when infusions of saline, acacia, or blood were given to dogs. Huckabee, Casten, and Harrison (14) reported heart failure in some of their dogs made hypervolemic with infusions of albumin, heparinized horse blood, or dog blood.

In previous reports from this laboratory (9,8) the effects of continuous intravenous infusions of gum acacia solutions were measured in dogs in which the chest was opened along the midline, the ventricles enclosed in a cardiometer, and respiration maintained by a positive-pressure pump. It was found that hypervolemia often led to acute heart failure. Because some of the effects observed might be related to the open chest, the acacia infusions were repeated in intact dogs. Preliminary results suggested (6,7) that the effects of infusion were similar in both types of experiment. This paper presents the results in more detail and attempts to correlate some of the responses of the cardiovascular system to the acute increase in the blood volume.

Methods

Normal, healthy dogs weighing between 9.5 and 22.0 kgm. were deprived of food, but not water, for 18 hr., and were then anesthetized by an intravenous

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injection of sodium pentobarbital. The usual dose was 30 mgm./kgm.; anesthesia was maintained by intramuscular or intravenous pentobarbital as required. No measurements of cardiac output were taken until two hours after the beginning of anesthesia, at which time the hematocrit value had usually returned to its preanesthetic level. During this period the minor operative procedures and other preparations necessary for taking measurements were completed.

The dog was placed on its right side. Two polyethylene catheters 20 in. long and 0.047 in. internal diameter were inserted into the left external jugular vein and introduced into the right ventricle, one then being withdrawn into the right atrium. The catheters were attached to saline manometers to measure mean right auricular pressure (RAP) and right ventricular pressure (RVP). All pressure readings were expressed from the estimated level of the right auricle, which was arbitrarily chosen as 5 cm. above table level. Blood clots were prevented from forming at the tips of the catheters by occasional flushing of the system with 1 or 2 ml. of saline.

The mean arterial blood pressure was recorded on a kymograph by a mercury manometer attached to a cannula in the right brachial or radial artery. Pulse rates were measured from the blood pressure tracing.

The direct Fick method was used for determination of the cardiac output. Oxygen consumption was measured with a Benedict-Roth spirometer connected to a large-bore plastic tube introduced into the trachea. The tube was held in position by a thin rubber collar which was inflated; thus no air could pass into or out of the lungs except through the endotracheal tube. Blood samples for the determinations of cardiac output were withdrawn over a one- to two-minute period during measurements of the oxygen consumption, but not until the animal had been breathing pure oxygen for at least five minutes. The arterial blood sample was obtained from the arterial cannula, and the mixed venous sample from the right ventricular catheter. The first few milliliters of blood were always discarded before the samples for gas analysis were taken. Five milliliters of blood were withdrawn into greased syringes containing 1–2 mgm. powdered heparin. A few drops of mercury were drawn into the syringe through a hypodermic needle, and the latter was plugged with a rubber stopper. The sample was then mixed, and the syringe placed in a water bath at about 2° C. until the time of analysis. Analyses were done in duplicate by the method described in Peters and Van Slyke (21), usually within one-half hour after withdrawal.

Samples for hematocrit determinations were taken from the arterial cannula and measured, in duplicate, in Wintrobe tubes centrifuged at 2500 r.p.m. for 30 min.

The calf and paw blood flows were measured by means of the venous-occlusion technique of Barcroft and Edholm (1) using specially-designed plethysmographs and water or air transmission to Brodie float-recorders. The calf blood flow represented muscle blood flow primarily, whereas the paw blood flow represented chiefly the skin blood flow; details of this technique have been described (13).

The infusion consisted of 6% gum acacia (B.D.H.) dissolved in 0.9% sodium chloride. This solution was buffered with sodium bicarbonate to pH 7.2 and filtered. After a control period of 30 to 120 min. a continuous infusion was made into a cannula in the cephalic or axillary vein. The rate of infusion was measured by a specially-built drop recorder using a relay and an impulse counter.

The intracardiac pressures, arterial blood pressure, pulse rate, infusion rate, and peripheral blood flows were measured every 5 to 10 min. throughout the experiments. Periodic estimations of oxygen consumption, A–V oxygen difference, and hematocrit were made. The cardiac output and percentage utilization of arterial oxygen (defined as $\frac{\text{A–V oxygen difference}}{\text{arterial oxygen content}} \times 100$) were calculated.

Experiments were performed in which the mean left auricular pressure (LAP) was also measured. The chest was opened under sodium pentothal anesthesia two to four days before the infusion day. A polyethylene catheter, filled with a solution of heparin in saline, was tied into the left atrium. The pericardium was then sewn up, and the catheter led out through the chest wall and clamped—later to be connected to a saline manometer. The incision was carefully closed, the lungs were re-expanded, and the dog was given penicillin to minimize infection. A chest Xray was taken 12–18 hr. before the experiment to determine the state of the lungs. It was found that in 7 out of 10 such animals the lungs were not grossly abnormal and the control cardiac outputs were within the same range as those of the intact dogs.

Results

A series of control experiments was performed to compare with the results of acacia infusion. The dogs were prepared exactly as described above, and measurements made over several hours of pentobarbital anesthesia; no infusion was given. The means and standard deviations of the various indices for five control dogs are shown in Table I. Control dogs recovered quickly and apparently completely.

TABLE I

CARDIOVASCULAR MEASUREMENTS IN CONTROL EXPERIMENTS

Dog	Weight (kgm.)	Hr. of anesthesia	No. of estimations	Cardiac output (l./min.), mean and S.D.	O_2 cons. (cc./min.), mean and S.D.	O_2 utilization, mean and S.D.	Hematocrit, %, mean and S.D.	Rt. auric. pressure (mm. saline), mean and S.D.
1	17.0	5	4	2.4 ± 0.31	121 ± 3.0	21.6 ± 2.8	48 ± 1.4	−0.2 ± 1.1
2	17.5	4	3	2.2 ± 0.25	93 ± 5.0	17.3 ± 2.2	57 ± 3.8	3.5 ± 1.7
3	18.7	4	3	2.2 ± 0.20	112 ± 10.0	25.0 ± 1.4	44 ± 2.7	1.5 ± 1.6
4	18.0	5	4	2.2 ± 0.12	130 ± 13.0	31.6 ± 1.0	45 ± 3.1	0.5 ± 1.0
5	17.7	5	4	2.2 ± 0.29	118 ± 5.0	23.0 ± 3.6	51 ± 2.8	2.7 ± 1.3

The most important findings in a typical infusion experiment are depicted in Fig. 1.

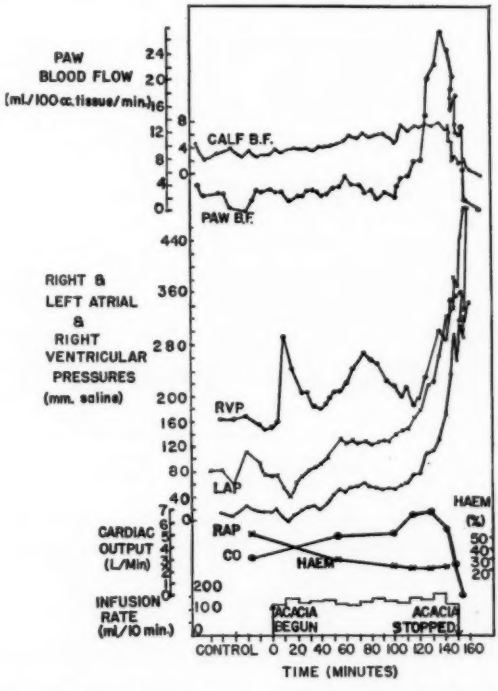

FIG. 1. Cardiovascular effects of gum acacia infusion; dog, 18 kgm., male. From above downwards: calf blood flow and paw blood flow in ml./100 gm. tissue/min.; right ventricular mean pressure, left auricular and right auricular mean pressures—all in mm. saline; hematocrit value in %; cardiac output in liters/min.; and infusion rate in ml./10 min. 1 ml. Nembutal I.M. at 60 and 120 min. Mean infusion rate in this dog was 1.0 ml./kgm./min. Note the large increase in cardiac output as the hematocrit level was reduced by infusion.

This figure shows the gradual rise in cardiac output and the decrease in hematocrit value which occurred as the volume of the circulation was increased by continuous intravenous infusion of acacia solution at an average rate of 1 ml./kgm./min. beginning at time "zero." The mean right auricular (RAP) and right ventricular (RVP) pressures increased slowly at first, then more rapidly as the cardiac output approached its maximum, and very abruptly as decompensation developed. The RVP-curve was frequently observed to have a saw-toothed character in these experiments. The calf and paw blood flows were seen to rise steadily with the cardiac output until the peak output was approached. At that time the paw blood flow increased sharply, while the calf blood flow continued to increase at its previous rate. When the heart began to fail, the blood flows decreased markedly, the paw flow falling almost

TABLE II

SUMMARY OF EFFECTS OF ACACIA INFUSION IN DOGS*

Expt. No. Wt. Sex	Time (min.)	Hct. value, %	Cardiac output (l./min.)	Coeff. O_2 util., %	Auric. press. (mm. saline)		Art. B.P. (mm. Hg)	TPR*	Blood flow (ml./100 gm. min.)		Volume acacia infused (ml.)
					RAP	LAP			Calf	Paw	
1	Control	54.0	3.2	25	56	—	140	2.62	2.6	1.4	0
	50	41.5	4.4	23	143	—	150	2.04	4.1	2.6	555
22 kgm.	75	37.5	5.3	30	140	—	150	1.70	4.8	3.5	826
	90	37.0	5.5	38	147	—	150	1.64	5.7	4.1	1016
Male	105	34.0	5.5	42	140	—	160	1.74	8.3	3.5	1222
2	Control	44.0	1.6	23	− 10	—	138	5.18	4.8	1.9	0
10 kgm.	40	27.5	4.1	15	40	—	162	2.38	9.3	8.1	425
Female											
3	Control	46.0	2.4	18	20	—	130	3.26	4.2	5.1	0
	10	41.0	2.5	18	42	—	144	3.46	5.4	13.2	79
16 kgm.	55	33.0	3.2	16	78	—	168	3.15	8.1	17.7	539
	100	27.5	5.0	16	122	—	160	1.92	7.2	16.8	1173
Male	135	20.0	5.7	18	157	—	152	1.60	9.7	12.0	1596
4	Control	40.0	2.1	26	− 12	—	—	—	—	—	0
	45	33.0	3.1	22	5	—	—	—	—	—	296
18 kgm.	100	24.0	3.8	24	98	—	—	—	—	—	772
Male	125	22.0	4.4	24	112	—	—	—	—	—	1005
5	Control	51.0	2.6	17	20	—	134	3.10	—	—	0
	55	34.0	3.1	28	117	—	125	2.42	—	—	473
15 kgm.	100	27.0	3.0	27	20	—	115	2.30	—	—	936
	155	19.0	3.3	34	− 15	—	85	1.55	—	—	1373
Female	190	15.0	4.9	26	20	—	100	1.22	—	—	1954
6	Control	46.0	1.4	25	− 1	—	80	2.91	1.8	2.0	0
	30	33.0	2.0	23	12	—	95	3.51	3.5	1.5	242
9.5 kgm.	90	23.0	2.7	27	24	—	165	3.67	10.2	8.3	723
Male											

7	Control	47.0	2.2	27	65	—	149	4.06	—	—	0
	50	28.0	4.9	20	160	—	172	2.11	—	—	1030
17 kgm.	85	28.0	4.6	25	176	—	178	2.32	—	—	1700
Female	175	16.0	5.6	36	250	—	162	1.74	—	—	3790
8	Control	49.0	2.4	23	− 30	—	124	3.10	—	—	0
15 kgm.	70	24.0	4.1	25	160	—	144	2.11	—	—	1330
Female	160	12.0	4.6	44	75	—	104	1.36	—	—	3050
9	Control	34.0	2.6	27	47	—	128	2.96	—	—	0
20.5 kgm.	60	26.0	3.7	30	140	—	152	2.46	—	—	1220
Female	115	19.0	5.8	29	134	—	148	1.53	—	—	2150
10	Control	50.0	1.6	36	30	70	82	3.08	—	—	0
11 kgm.	60	30.0	2.6	32	115	268	88	2.03	—	—	470
Female	130	28.0	3.0	30	130	350	74	1.48	—	—	1094
11	Control	49.0	4.5	16	0	10	92	1.23	4.5	1.0	0
	105	29.5	4.5	22	17	83	106	1.41	15.0	3.9	1107
16 kgm.	150	24.0	5.6	24	2	69	156	1.62	15.5	6.9	1653
Female	195	20.0	6.2	24	5	61	152	1.47	15.1	9.1	2308
12	Control	30.0	1.2	42	12	− 15	86	4.30	5.8	0.3	0
10 kgm.	35	19.0	3.5	26	58	245	120	2.06	9.9	0.8	375
Female	45	14.0	4.0	25	62	225	102	1.53	10.4	1.5	490
13	Control	50.0	2.9	21	17	90	130	3.69	2.5	1.5	0
	45	29.0	4.3	24	41	110	150	2.10	3.8	3.3	588
18 kgm.	95	24.0	4.5	26	50	135	150	2.00	5.4	3.4	1239
	110	22.5	5.9	22	70	160	158	1.61	6.4	6.3	1426
Male	126	21.5	6.0	23	125	253	122	1.22	7.0	24.4	1708
14	Control	52.0	1.4	45	9	83	130	5.58	1.6	2.4	0
14.5 kgm.	30	34.5	3.0	35	55	102	146	2.92	3.6	1.6	342
Male											
15	Control	52.0	3.2	25	− 10	5	100	1.88	1.4	4.1	0
22 kgm.	50	35.0	4.1	31	47	100	114	1.67	5.4	19.8	56
	100	32.5	6.0	24	106	107	110	1.10	6.8	22.0	1022
Male	130	29.5	7.0	22	144	145	110	0.94	7.0	26.5	1355

* *See text for amplification.*

to zero. Death occurred within twenty minutes of the onset of failure. (The term "failure" in this paper is defined as that point at which the cardiac output began to decrease and continued to decrease while the RAP continued to rise.)

A summary of the most important cardiovascular effects in 15 dogs of gum acacia infusions is given in Table II. This table shows the experimental number, weight, and sex of the dog, the average control values of all the variables, the time in minutes from the beginning of infusion, the hematocrit value (in %), the cardiac output (in liters/min.), the coefficient of oxygen utilization (in %), the auricular mean pressures (in mm. saline), the arterial mean pressure (in mm. Hg), the total peripheral resistance (calculated by dividing the arterial mean pressure in mm. Hg by the cardiac output in ml./second), the calf and paw blood flows (in ml./100 gm. tissue/minute), and the volume (in ml.) of acacia infused at the time shown. Although the intracardiac and arterial pressures, peripheral blood flows, and volume infused were measured every 10 min. throughout the experiments, only those coinciding with cardiac output determinations are shown in this table. No results obtained after the peak output had been reached, or after administration of a drug (other than the anesthetic) are shown.

The table shows that acacia infusion always produced a decrease in the hematocrit value. In every experiment the cardiac output was increased, and in many this increase was more than 100% of the control level. The arterial blood pressure usually increased with infusion, but the cardiac output rose whether the arterial pressure increased or not. Changes in pulse rate were less than 10% of the control values, and thus the increased cardiac output was primarily an increase in stroke output. As the cardiac output increased, the total peripheral resistance regularly decreased and remained below control values until the heart began to fail, at which time the TPR increased. Both the paw and calf blood flows increased as the cardiac output rose, and both tended to level off as the peak output was reached. The changes in resistance, calculated from the plethysmographic data, in both paw and calf followed the same general pattern as the TPR. When cardiac failure occurred, the paw blood flow decreased much faster than the calf flow.

The oxygen consumption did not change significantly during the infusion (average increase 6.5%), but the A–V oxygen difference became progressively less, until at the maximal output it had decreased by an average of 42% $\pm$ 5.6 (S.E.M.). Although the arterial oxygen content decreased as the hematocrit value fell, the percentage of oxygen extracted from the arterial blood remained within normal limits until the heart began to fail; with failure the oxygen utilization consistently increased.

The table also shows that the right auricular mean pressure rose progressively with the cardiac output in some experiments, whereas in others (Nos. 5, 8, 11) it increased early in the infusion, and then decreased while the cardiac output continued to rise. When LAP was also measured, it usually began to rise sooner than the RAP and/or at a more rapid rate.

The volume of acacia solution infused up to the time of the peak cardiac output was found to vary greatly in these experiments.

On gross examination post mortem a greatly dilated heart and distended systemic veins were regularly found. Occasionally there were a few small subpericardial hemorrhages; the coronaries were invariably patent. Gross pulmonary edema was not evident, but the lungs often showed moderate congestion. There was frequently a clear, straw-colored pleural effusion. The liver was engorged, the spleen was contracted, and occasionally there were small subserosal hemorrhages in the bowel. Gross examination showed nothing abnormal in the kidneys. There were often 100–200 ml. of clear ascitic fluid; marked peripheral edema, sometimes pitting, was occasionally observed.

In this type of experiment the degree of dilution anemia brought about by acacia infusion is measured more precisely by the hematocrit levels than by the volume or duration of infusion because of the differing infusion and excretion rates. Therefore the cardiovascular changes were compared at various hematocrit levels. The coefficient of correlation between the increases in cardiac output (percentage increase over control levels) and the hematocrit values was -0.53, whereas the coefficient between the increases in cardiac output and the right auricular mean pressures was only 0.36.

Discussion

From the control experiments it was found that if the level of anesthesia was maintained relatively constant throughout the experiment, the cardiac output showed only minor changes after the first two hours of anesthesia. This is in accord with previous reports (22, 32) of the cardiac output in dogs anesthetized with pentobarbital. The rapid and marked increases in output observed during gum acacia infusion were thus undoubtedly related to the infusion. The increased cardiac output following infusions of saline, plasma, dextran, and polyvinylpyrollidone which we have observed in other studies indicate that the increases occurring here are not due to any peculiar property of gum acacia.

In every experiment the cardiac output was increased by the infusion of gum acacia solution; this is in agreement with various other reports (3, 8, 10, 13, 14, 26, 29) of increased cardiac output accompanying both acute and chronic anemia in animal experiments and in man. In some of our experiments the cardiac output increased by more than one hundred per cent over control levels before failure occurred. When the arterial oxygen content was reduced by dilution of the blood with acacia, the demands of the tissues for oxygen apparently were met by an increased cardiac output maintaining the arterial oxygen transport, and not by an increased extraction of oxygen. In fact, the percentage utilization of arterial oxygen usually remained within normal limits until cardiac decompensation occurred. When the heart

began to fail, a much greater percentage of oxygen was removed from the arterial blood, but even then it appeared that oxygen demands were not being met.

From a study of acute hypoxemia in dogs Feldman, Rodbard, and Katz (5) concluded that their results were in accord with other findings that the cardiac output is a function of the tissue oxygen requirements, and that when tissue hypoxia exists, the cardiac output will rise as a compensatory mechanism. When the lack of oxygen becomes extreme, the heart and central nervous system begin to weaken and the cardiac output falls, probably because of heart failure. Halmagyi *et al.* (12) thought that a simple elevation of circulating plasma volume (produced by large intravenous infusions in dogs) did not create a situation similar to human congestive heart failure. They considered that whereas the former is a simple mechanical distension of the venous system, the latter is a true venous hypertension being due to an active participation of the nervous system.

Plethysmography showed that with infusion both the calf and paw blood flows increased markedly. This occurred even though no consistent changes were observed in arterial blood pressure and the central venous pressure was gradually increasing. Thus it is obvious that infusion produced a large decrease in total peripheral resistance. Near the point of failure, however, the total peripheral resistance increased and blood flows decreased; the calf flow fell slowly, the paw flow rapidly.

The fact that the left auricular mean pressure usually increased at a more rapid rate than the right is to be expected from the pressure–volume curves obtained by Little (16) in isolated dog hearts with the A–V valves sutured. He found that the right atrial system required twice the volume of the left for initial filling, and that even when filled, the right atrium was much more distensible than the left and its pressure was lower. Cournand (4) also drew attention to the marked differences in capacity and distensibility of the two venoatrial systems.

The regression lines calculated for changes in cardiac output and right auricular pressure show that in these experiments the cardiac output is governed less by the RAP than by other factors. In several experiments the RAP actually decreased while the cardiac output continued to rise. This dissociation between RAP and cardiac output has been noted by other workers. Stead and Warren (28) found that increases in RAP of as much as 100 mm. water (rapid infusions of saline and albumin solutions into normal subjects) produced no constant changes in cardiac output. In a later review Stead (27) stated that changes in RAP do not cause the consistent changes in cardiac ouput which are seen in the heart–lung preparation, and he added that the increased cardiac output of anemia, thyrotoxicosis, apprehension, and sudden lowering of the peripheral resistance are not accompanied by increases in RAP. Sharpey-Schafer (26) reported that the cardiac output in anemia was elevated at rest, but the RAP was not unless cardiac failure was present. Others (2, 23) found that drugs such as paredrinol and angiotonin would

increase the RAP but not the output; McMichael and Sharpey-Schafer (18) and Stead and Warren (28) reported that adrenaline produced an increase in stroke volume, but at the time of this increase the atrial pressure was not changed or even fell. In muscular exercise although the venous pressure, cardiac output, and stroke volume increased, Nylin (20) found that the cardiac size diminished. Sunahara and Beck (29) found no consistent relation between cardiac output and RAP in their study of acute anemia in dogs, and Gregg (11) concluded from his experiments that the atrial or end diastolic ventricular pressure did not exercise any dominant control over stroke volume in the intact normal animal. Others (15) have shown that the maximum outputs in preparations such as those studied by Starling never reach even the level found at rest in an intact animal of the same size. On the other hand, McMichael and Sharpey-Schafer (18) showed that with intravenous infusions of saline both RAP and cardiac output increased, and Huckabee *et al.* (14) obtained similar results in dogs with infusions of saline, albumin, and blood. McMichael (17) states that the influence of the venous filling pressure of the heart in determining the diastolic volume, and thereby the capacity of the stretched fibers for work, is a fundamental part of Starling's law; the cardiac output of the normal heart rises with an increase, and falls with a decrease, of venous pressure. Sarnoff (25) concluded from his experiments on open-chest dogs that Starling's Law of the Heart could be verified experimentally, that the filling pressure in the right auricle and the left auricle may not be related (each ventricle may function on a different level of performance), that there exist several Starling's curves for each heart chamber, and that the heart will function along one or another of these depending on the momentary determinants.

In reviewing this whole question of the applicability of Starling's law to the intact circulation Richards (24) concluded that apparently while there is a tendency in the normal circulation to follow Starling's law, there are also influences of nervous or metabolic origin which may alter such functions as cardiac tone (diastolic heart size at a given inflow pressure) or systolic emptying and thus modify cardiac performance outside this law.

The experiments described in this paper show that although right auricular pressure may play a role in the increased output produced by infusion of gum acacia, other factors such as arterial oxygen capacity are of greater importance.

Acknowledgments

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STUDIES ON THE PRESERVATION OF BLOOD

II. THE GLYCOLYTIC BEHAVIOR OF BLOOD DURING STORAGE[1]

By Hanna M. Pappius[2] and Orville F. Denstedt

Abstract

The chemical changes that occur in erythrocytes when blood is admixed with isotonic sodium citrate and stored at 5° C have been studied. When the residual glucose content of the red cells is depleted, about the fifth day of storage, the continuity of the glycolytic system is interrupted, as indicated by an abrupt decrease in the concentration of diphosphoglycerate and a rise in pyruvate. These changes can be retarded by the addition of glucose to the preservative medium at the beginning of storage or at any time before the residual glucose content becomes depleted. The nature and sequence of the chemical changes that occur during storage are the same whether the blood specimen is preserved in the defibrinated state or in the presence of citrate. Also these changes are the same, except in rate, at temperatures between 5° and 37° C.

Introduction

In a previous paper (4) the authors discussed the glycolytic behavior of citrated blood stored at 5° C. with a moderate excess of glucose in the samples. The present communication describes the glycolytic activity of citrated and of defibrinated blood at various temperatures without the addition of glucose to the specimens. The object of the study was threefold: (1) to study the effect of the depletion of the blood glucose on the glycolytic behavior of the erythrocyte; (2) to ascertain whether the glycolytic activity of the red cells is influenced by the presence of citrate in the external medium; (3) to ascertain whether storage at various temperatures alters the glycolytic system other than by influencing the rate of utilization of glucose and of production of lactic acid.

Experimental

Methods

The procedure for the collection and storage of blood, and the details of the analytical methods, were as described in a previous communication (4). In most of the experiments the blood was collected in isotonic citrate solution (3.2% trisodium citrate), the ratio of the volume of blood to citrate solution being 5 : 2 in all instances.

Blood specimens which were collected without the addition of citrate were defibrinated aseptically by stirring the specimen slowly for about 20 min. in a gauze-covered flask with a whisk of applicator sticks. The whisk, with the retracted fibrin, was removed and the flask was closed with a sterile stopper.

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Contribution from Department of Biochemistry, McGill University Montreal, Que. The material in this paper is taken from theses submitted by H. M. Pappius in partial fulfillment of the requirements for the degree of Master of Science (1948) and of Doctor of Philosophy (1952) in Biochemistry at McGill University.

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The glycolytic activity of the various blood specimens was studied during storage at 5° ± 1°, 20°, and 37°, respectively.

Results

Glycolytic Behavior of Citrated Blood During Storage at 5° C.

The results illustrated in Fig. 1 are typical of the findings in seven experiments.

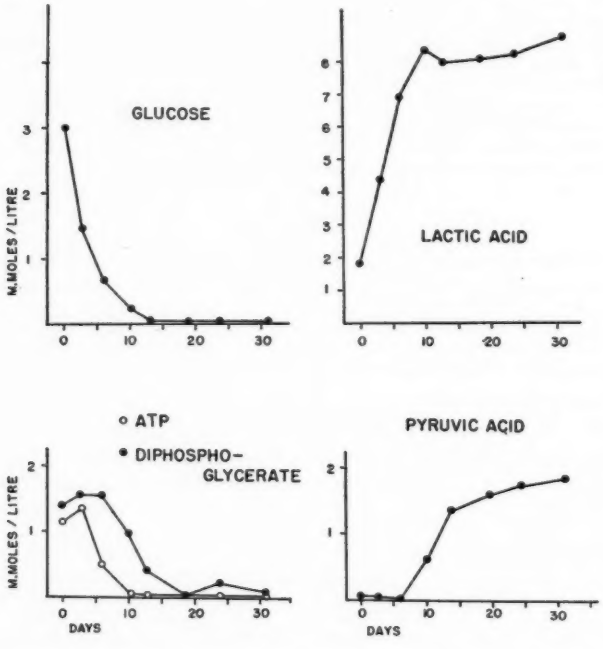

FIG. 1. Glycolytic changes in blood stored in isotonic citrate medium at 5° C. Proportion of blood to 3.2% trisodium citrate of 5 : 2 by volume.

The glucose content of citrated blood at 5° decreased steadily from the beginning of storage and approached zero about the end of the first week. Concurrently with the fall in glucose, the lactate concentration was increased while the concentration of pyruvic acid and diphosphoglycerate remained practically unchanged. With the depletion of glucose, however, the concentration of diphosphoglycerate began to fall and the pyruvate content abruptly to rise. The rate of accumulation of pyruvate was diminished as the concentration of diphosphoglycerate, its precursor, approached zero about the second week of storage. The concentration of ATP decreased steadily after the first three days of storage, and approached zero about the 10th day.

The changes in the concentration of the total acid-soluble phosphate, inorganic phosphate, and organic acid-soluble phosphate are set forth in Table I.

TABLE I

THE BEHAVIOR OF THE VARIOUS PHOSPHATE FRACTIONS IN CITRATED BLOOD DURING STORAGE AT 5° C.

Duration of storage, days	Total acid-soluble phosphate	Inorganic phosphate	Organic acid-soluble phosphate
	Millimoles PO_4 per liter of whole blood		
0	5.21	1.21	4.00
3	5.37	1.00	4.37
6	5.21	1.47	3.74
10	5.21	3.10	2.11
13	5.21	4.37	0.84
19	5.21	5.58	—
24	5.32	5.32	—
31	5.26	5.79	—

NOTE: *Proportion of blood to 3.2% trisodium citrate solution of 5 : 2 by volume.*

The constancy of the total acid-soluble phosphate in all the samples indicates the uniformity of the red cell content and accuracy of sampling. The concentration of inorganic phosphate remained practically constant during the first week of storage, then began to increase, reaching the maximum value at the end of the second week. Thereafter, the concentration of inorganic phosphate remained unchanged. The change in the concentration of the organic acid-soluble phosphate, as represented by the difference between the total acid-soluble phosphate and the inorganic phosphate, varies inversely with the concentration of inorganic phosphate.

The behavior of the various compounds as shown in the figure and the table is characteristic of blood specimens stored in isotonic citrate solution. The only difference noted in the behavior of the individual preserved blood specimens was with respect to the time when the pyruvate concentration began to increase and the diphosphoglycerate to fall. These changes occurred usually between the fifth and ninth days of storage, and always coincided with, and therefore probably were initiated by, the depletion of the residual glucose in the sample.

Glycolytic Behavior of Defibrinated Blood During Storage at 5° C.

To ascertain whether the citrate, used as anticoagulant, has any direct or indirect influence on the glycolytic behavior of the red cells during storage the experiments were repeated with defibrinated specimens.

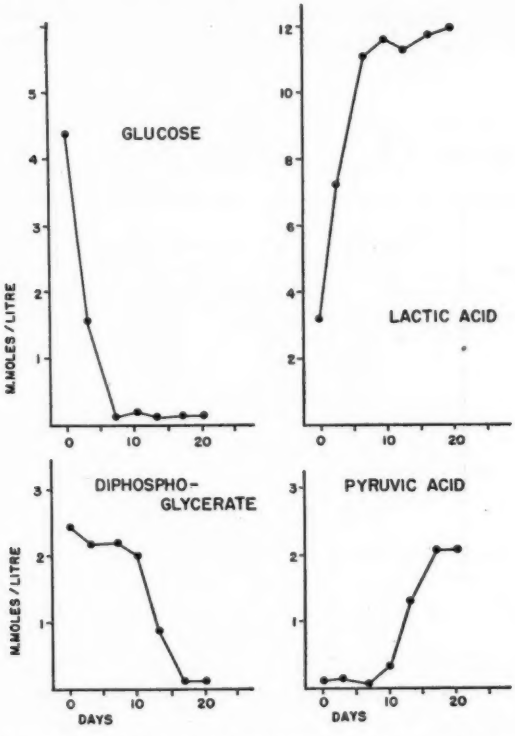

FIG. 2. Glycolytic changes in defibrinated blood stored at 5° C.

TABLE II

THE BEHAVIOR OF VARIOUS PHOSPHATE FRACTIONS OF DEFIBRINATED BLOOD DURING STORAGE AT 5° C.

Duration of storage, days	Total acid-soluble phosphate	Inorganic phosphate	Organic acid-soluble phosphate	Hydrolyzable phosphate
	Millimoles PO_4 per liter of whole blood			
0	7.57	0.68	6.89	2.00
3	7.73	0.68	7.05	2.68
7	7.78	1.05	6.73	2.42
10	7.94	2.84	5.10	1.11
13	8.00	5.05	2.95	1.16
17	7.74	6.84	0.90	0.58
20	7.73	7.21	0.52	0.27
25	7.37	7.73	0.00	0.00

It may be observed from the results in Fig. 2 and Table II that the metabolic behavior of the red cells in the defibrinated samples was practically the same as that of the citrated specimens when allowance is made for the differences in the dilution of the red cells. The character and sequence of the changes were the same whether the red cells were stored in the defibrinated or in citrated plasma.

Glycolytic Behavior of Citrated and Defibrinated Blood Specimens During Storage at 20° and at 37° C.

To compare the glycolytic behavior of blood at higher temperatures with that at 5° experiments similar to those already discussed were repeated with storage temperatures of 20° and 37°.

Fig. 3 illustrates the changes in the concentration of glucose, lactate, diphosphoglycerate, and pyruvate in citrated and defibrinated blood specimens kept at 20°.

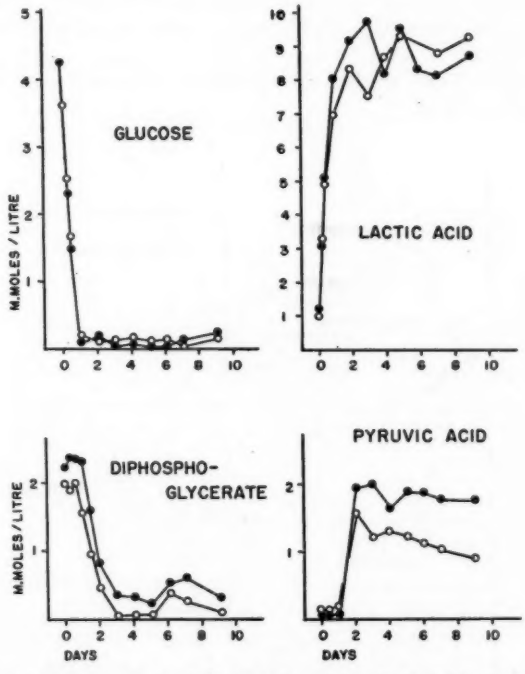

FIG. 3. Glycolytic changes in defibrinated and in citrated blood stored at 20° C. Proportion of blood to 3.2% trisodium citrate in citrated blood of 5 : 2 by volume. ○ Citrated blood. ● Defibrinated blood.

It is evident that the pattern of the glycolytic changes in the two samples during storage differed little from that observed at 5°, except that the rate of change was considerably accelerated at the higher temperature. Thus in

both samples the glucose was rapidly utilized and approached zero concentration within 24 hr. Concurrently the concentration of lactate increased steadily until the glucose was exhausted. The concentration of diphosphoglycerate and of pyruvate, however, remained unchanged. Coincident with the depletion of the glucose the level of diphosphoglycerate began to fall and the concentration of pyruvate to rise steeply both in the citrated and the defibrinated samples. The only difference in the behavior of the two blood samples was in the behavior of pyruvate during the last part of the experiment. Thus, in the defibrinated specimen little change in the concentration of pyruvate had occurred by the second day of storage, and the concentration of lactate had already levelled off. In the citrated sample, on the other hand, the concentration of pyruvate underwent a gradual decrease after the second day while the concentration of lactate was increased.

No significant difference was observed between the behavior of the various phosphate fractions in the defibrinated blood and the citrated specimens stored at 20°. The chemical changes during storage, in both cases, were analogous to those observed at 5°, apart from the accelerated rate at the higher temperature.

The chemical changes during the storage of citrated blood samples at 37° are illustrated in Fig. 4.

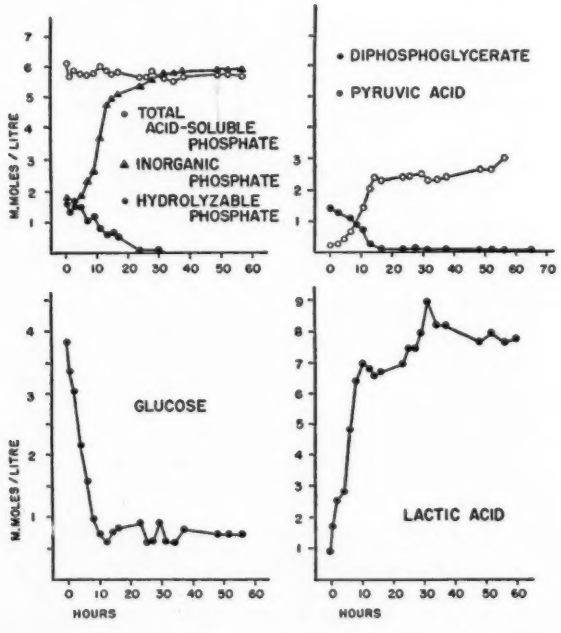

FIG. 4. Glycolytic changes in citrated blood incubated at 37° C. Proportion of blood to 3.2% trisodium citrate solution, 5 : 2.

It is evident that the general chemical behavior of blood during storage at 37° is similar to that at 5° and at 20°, apart from the increased rate of change with the increase of the temperature. Comparing the behavior of pyruvate in the samples at the three temperatures it may be seen that at 37° a slow increase in the concentration of pyruvic acid apparently occurred from the beginning of the experiment and that the rate of accumulation of the pyruvate was increased after most of the glucose had been used up.

Effect of Adding Glucose to Preserved Blood Specimens at Various Stages During Storage on Chemical Changes in Citrated Blood

Since the abrupt change in the concentration of pyruvate and of diphosphoglycerate in blood preserved in citrate occurred when the glucose was depleted, it was of interest to test the effect of replenishing the glucose in the preserved specimens at various times. In these experiments a sample of blood collected in citrate–glucose medium was included as a control.

Fig. 5 indicates the effect of adding glucose to citrated blood on the 5th, 10th, 14th, and 20th day, respectively, of storage at 5°. The analytical results are tabulated in Tables III, IV, V, and VI.

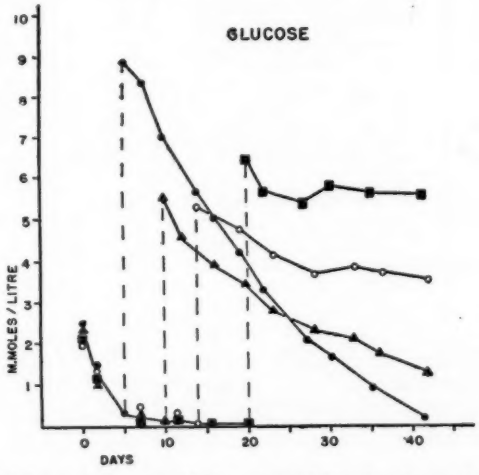

FIG. 5. Breakdown of glucose added to citrated blood at intervals of storage.

● Glucose added on 5th day of storage
▲ " " " 10th " " "
○ " " " 14th " " "
■ " " " 20th " " "

It may be seen that when glucose was added to the citrated blood on the fifth day of storage, just before the residual glucose was depleted, the added sugar was metabolized at the rate at which the residual sugar had been utilized during the previous five days, or at which the added sugar was glycolyzed when added to the preservative medium at the beginning of storage. In the

TABLE III

THE EFFECT OF ADDING GLUCOSE TO CITRATED BLOOD AT VARIOUS TIMES DURING STORAGE AT 5° C.

Duration of storage, days	Sample*				
	A	B	C	D	E
	Day of addition of glucose				
	5	10	14	20	0
	Millimoles of glucose per liter of blood				
0	2.55	2.30	2.36	2.16	19.44
2	1.39	1.11	1.22	1.17	—
5	0.44	—	—	—	18.44
—	8.94	—	—	—	—
7	8.44	0.28	0.22	0.17	—
10	7.00	0.14	—	—	—
—	—	5.55	—	—	—
12	—	4.61	0.17	0.17	—
14	5.74	—	0.14	—	15.33
—	—	—	5.36	—	—
16	—	4.07	5.17	0.03	—
19	4.33	—	4.83	—	14.05
20	—	3.58	—	0.08	—
—	—	—	—	6.50	—
22	3.33	—	—	5.75	—
23	—	2.86	4.20	—	12.22
27	2.17	—	—	5.44	—
28	—	2.47	3.78	—	11.33
30	1.80	—	—	5.89	—
33	—	2.17	3.94	—	12.00
35	1.03	—	—	5.78	—
36	—	1.92	3.89	—	11.55
41	0.30	—	—	5.72	—
42	—	1.47	3.69	—	—

* *Samples A–D were collected in isotonic citrate solution (3.2% trisodium citrate) in proportion by volume of blood to citrate of 5 : 2. Isotonic glucose solution was added on appropriate days to raise the glucose content of the mixture to between 8 and 5 mM. per liter. Sample E was collected into isotonic citrate–glucose mixture in proportion by volume of blood to citrate to glucose of 5 : 2 : 0.5.*

samples to which glucose was added on the 10th, 14th, and 20th day, respectively, the rate of utilization of the sugar became progressively slower as the interval before replenishment of the sugar was increased. This illustrates how the activity of the enzymes becomes impaired in the absence of substrate and the progressively decreased effectiveness of the added sugar in restoring the activity of the system. The total production of lactic acid in all samples corresponded closely to the amount of glucose broken down in the red cells during storage.

The behavior of the diphosphoglycerate in the samples to which glucose was added on the fifth day, and in the specimen which had been stored in

TABLE IV

THE EFFECT OF ADDING GLUCOSE ON THE BEHAVIOR OF LACTIC ACID IN CITRATED BLOOD DURING STORAGE AT 5° C.

Duration of storage, days	Sample*				
	A	B	C	D	E
	Day of addition of glucose				
	5	10	14	20	0
	Millimoles lactic acid per liter of blood				
0	1.77	1.61	1.66	2.11	2.16
2	3.83	4.16	4.05	3.50	—
5	5.88	—	—	—	7.11
7	7.22	6.33	7.05	6.61	—
10	11.25	7.61	—	—	—
12	—	7.94	7.33	7.27	—
14	13.47	—	7.92	—	14.72
16	—	9.58	8.33	6.94	—
19	15.97	—	9.58	—	16.94
20	—	10.69	—	7.22	—
22	16.11	—	—	7.50	—
23	—	11.11	8.88	—	18.33
27	18.33	—	—	7.50	—
28	—	12.32	8.19	—	18.88
30	20.83	—	—	9.30	—
33	—	13.47	9.58	—	21.11
35	22.36	—	—	8.47	—
36	—	14.86	10.14	—	23.61
41	24.30	—	—	9.58	—
42	—	15.38	10.00	—	23.05

* *See footnote Table III.*

citrate–glucose medium from the beginning was the same. In both cases, the time at which the concentration of diphosphoglycerate began to fall during storage was delayed and the rate of fall retarded, in contrast to the time at which the corresponding changes occurred in the samples to which glucose was added after the fifth day. Comparing the behavior of the blood specimen preserved in citrate alone with that of the sample stored from the beginning in citrate–glucose, the influence of the glucose in delaying the fall in the diphosphoglycerate and the rise in pyruvate is clearly evident. When the addition of glucose to the citrated blood specimen was delayed until the 10th, 14th, and 20th day, respectively, no significant resynthesis of the diphosphoglycerate fraction occurred, but it is noteworthy that in all three samples, the residual small concentration of the stable phosphate was maintained at a constant level throughout the remainder of the experimental period.

TABLE V

THE EFFECT OF ADDING GLUCOSE ON THE BEHAVIOR OF DIPHOSPHOGLYCERATE IN CITRATED BLOOD DURING STORAGE AT 5° C.

Duration of storage, days	Sample*				
	A	B	C	D	E
	Day of addition of glucose				
	5	10	14	20	0
	Millimoles diphosphoglycerate per liter of blood				
0	1.37	1.26	1.29	1.23	1.21
2	1.47	1.47	1.42	1.26	—
5	1.42	—	—	—	1.21
7	1.23	1.05	1.13	1.23	—
10	1.10	0.39	—	—	—
12	—	0.16	0.26	0.21	—
14	0.76	—	0.23	—	0.93
16	—	—	0.08	0.10	—
19	0.34	—	0.15	—	0.44
20	—	0.18	—	0.08	—
22	0.31	—	—	0.00	—
23	—	0.10	— 0.15	—	0.26
27	0.31	—	—	0.39	—
28	—	0.18	0.24	—	0.29
30	0.00	—	—	0.08	—
33	—	0.16	0.23	—	0.21
35	0.26	—	—	0.24	—
36	—	0.18	0.18	—	0.18
41	0.15	—	—	0.05	—
42	—	0.29	0.18	—	0.13

* *See footnote Table III.*

The addition of glucose to the citrated sample on the fifth day of storage, that is, before the residual glucose was depleted, was effective in delaying the onset of pyruvate accumulation, though not quite as effective as when the sugar was added at the beginning of storage. The addition of glucose on the 10th day not only interrupted the accumulation of pyruvate but led to a decrease in the concentration. The addition of sugar on the 14th day and on the 20th day, that is, after the pyruvate had reached the maximum concentration, had little effect, but the pyruvate level tended to fall slightly thereafter in the latter specimen.

Discussion

The glycolytic changes in citrated blood during storage can be satisfactorily explained by the modern theory of glycolysis. Apart from the difference in the rate at various temperatures the glycolytic behavior of the erythrocytes

TABLE VI

THE EFFECT OF ADDING GLUCOSE ON THE BEHAVIOR OF PYRUVIC ACID IN CITRATED BLOOD DURING STORAGE AT 5° C.

Duration of storage, days	Sample*				
	A	B	C	D	E
	Day of addition of glucose				
	5	10	14	20	0
	Millimoles pyruvic acid per liter of blood				
0	0.045	0.034	0.040	0.034	0.040
2	0.057	0.045	0.068	0.051	—
5	0.068	—	—	—	0.062
7	0.102	0.528	0.375	0.386	—
10	0.369	1.194	—	—	—
12	—	1.250	1.421	1.392	—
14	0.648	—	1.563	—	0.523
16	—	1.222	1.478	1.478	—
19	0.852	—	1.478	—	0.966
20	—	0.909	—	1.506	—
22	0.824	—	—	1.250	—
23	—	0.682	1.392	—	0.966
27	0.795	—	—	1.307	—
28	—	0.454	1.450	—	1.194
30	0.881	—	—	1.364	—
33	—	0.625	1.620	—	1.421
35	0.938	—	—	1.222	—
36	—	0.597	1.620	—	1.535
41	1.079	—	—	0.966	—
42	—	0.625	1.676	—	1.846

* *See footnote Table III.*

between zero and 37° follows the same pattern. As long as glucose is present glycolysis proceeds in the normal manner and lactate accumulates, but when the residual sugar is depleted, about the fifth day, the continuity of the glycolytic mechanism becomes impaired through alteration of the triosephosphate dehydrogenase and the lactic dehydrogenase systems which are DPN-linked. Thus when glucose is depleted, no more phosphoglyceraldehyde is formed and consequently no reduction of the DPN^+ can occur, nor can more diphosphoglycerate be formed. The conversion of the residual diphosphoglycerate to pyruvate, however, continues, but, since reduced DPN (DPNH) is in short supply, the conversion of pyruvate to lactate cannot occur and thus pyruvate accumulates.

In this connection it is interesting to note that the rate of accumulation of pyruvate and of breakdown of diphosphoglycerate is more rapid in citrated blood than in the specimens preserved in the citrate–glucose medium. In the latter the changes occur later during storage (4). It appears, therefore,

that in blood specimens preserved in citrate alone these changes are initiated by the depletion of the residual substrate, while in the specimens containing added sugar the changes are attributable to a progressive failure of the glycolytic mechanism from another cause.

Closely connected with the conversion of diphosphoglycerate to pyruvate in the erythrocyte is the regeneration of ATP. Coincidently with the depletion of the residual glucose and the fall in the diphosphoglycerate in citrated blood the concentration of ATP also begins to fall, and the inorganic phosphate from the breakdown of both these esters steadily accumulates until they are depleted. Whether under these conditions ATP is not formed during the breakdown of the residual diphosphoglycerate, or whether it is used up more rapidly than it can be formed, has not been settled. From thermodynamic considerations it is likely that only one high-energy phosphate bond is formed during conversion of 2,3-diphosphoglycerate to pyruvate (4).

In citrated blood specimens stored at 37° the fall in the concentration of diphosphoglycerate and the rise in pyruvate appear to proceed from the beginning of storage. At the same time there is a tendency for hemoglobin to undergo oxidation slowly to methemoglobin (2). Since the latter transformation is reversible and the reduction of the methemoglobin in the red cell is known to require DPNH (3), it is reasonable to suppose that some of the reduced coenzyme may be utilized for this purpose thus depriving the lactic dehydrogenase of its full quota of the coenzyme and favoring the early accumulation of pyruvate. Methemoglobin is not formed in red cells stored at 5° (1); indeed, any residual methemoglobin that may be present in the fresh blood usually undergoes reduction to hemoglobin shortly after being stored.

It is clear from Fig. 5 and Tables III to VI that the depletion of the residual glucose in the red cell in citrated blood specimens leads to an early and irreversible impairment of the glycolytic mechanism. If glucose is added at the beginning of the storage period or at any time prior to the complete exhaustion of the residual glucose, the signs of impairment, notably the fall in diphosphoglycerate and accompanying rise in pyruvate, are delayed until the 12th to 15th day of storage. The addition of glucose to the specimen after the residual glucose has been depleted tends to stabilize the system for a time at the existing level of activity but does not increase the activity. The addition of glucose does not cause an increase in the level of diphosphoglycerate or a fall in pyruvate. Thus in the absence of substrate some labile part of the glycolytic system suffers a progressive and irreversible impairment, which may be temporarily retarded but not prevented by the addition of glucose to the sample. In other words, while the depletion of substrate is undoubtedly the main cause of the fall in the concentration of diphosphoglycerate and the accumulation of pyruvate in citrated blood during storage, another process evidently is going on at the same time which tends to impair the activity of the glycolytic system since the same type of failure ultimately occurs even when glucose is present.

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NUTRITION OF ANIMAL CELLS IN TISSUE CULTURE

VII. USE OF REPLICATE CELL CULTURES IN THE EVALUATION OF SYNTHETIC MEDIA[1]

By Raymond C. Parker, George M. Healy, and Dorothy C. Fisher

Abstract

The replicate culture assay procedures of Earle and his associates have been adapted for use in evaluating the effectiveness of synthetic media. For this purpose, use has also been made of Earle's L strain mouse cells. Washed and continuously stirred suspensions of these or similar strains of cells may be dispensed, with reasonable assurance of uniformity, into a series of replicate cultures, the number depending on the volume of the suspension and the capacity and effectiveness of the stirring and dispensing unit. For use with synthetic media, the original procedures for the preparation and care of the replicate cultures and for their subsequent treatment for the counting of isolated, stained nuclei have been modified considerably. This paper describes the procedures that were finally adopted and also describes a relatively simple screening procedure in which washed cell suspensions may be used to advantage in making preliminary assays of synthetic media and in testing the relative toxicity or growth stimulating effects of substances added to, or derived from, natural media.

Introduction

Over the past six years, a continuous attempt has been made in this laboratory to develop a medium of chemically defined composition that will permit the unlimited multiplication and survival of animal cells in tissue culture. As the work has progressed, synthetic media of increasing complexity have been devised by the systematic addition of known growth factors and metabolites to a balanced salt solution containing glucose (9). In practice, each substance or group of substances under investigation is tested at various levels in order to determine the concentration at which it may be beneficial, or at least nontoxic. Until a year ago, these assays were made in roller tubes containing chick mesenchyme tissues cultivated directly on the surface of the glass. Each culture was prepared from the least amount of tissue that would provide adequate growth areas; and the media were withdrawn and replaced by fresh samples twice a week (10). The cultures were examined frequently under the microscope, and the effect of each mixture was determined by the length of the average period of survival of the tissue cells. Ordinarily, 12 or more cultures were tested at each concentration of the substances under investigation.

By the time Solution No. 199 had been developed (9), the medium contained 60 ingredients and supported cell life for an average period of 33 days, though odd cultures that chanced to contain even minute fragments of bone or cartilage continued to live for as long as 170 days (1). Eventually, in order to avoid such wide variations, heart muscle was substituted for the skeletal muscle that had been used previously. Also, as a means of speeding up the

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assays, the *average survival period*, as a measure of the relative effectiveness of the different media, was abandoned in favor of a new criterion, the *50% survival period*, at the end of which time only 50% of the cultures had succumbed, but the experiment could be terminated.

Over a year ago, the roller-tube method was discontinued as a routine means of screening new compounds. Because these assays took longer and longer to complete as improvements were made in the medium, it became increasingly difficult to study new compounds efficiently by these means alone. It was decided, therefore, to supplement these long-term roller-tube assays with an elaborate system of short-term replicate culture procedures devised by Earle and his associates (4, 12) at the National Cancer Institute, in Bethesda. According to these procedures, the number of cells placed in each of a series of cultures is estimated by counting the cell nuclei in representative samples of a washed and continuously stirred cell suspension used as inoculum. The samples are prepared for counting by treating the cells with citric acid followed by low speed centrifugation and light staining with crystal violet. The liberated nuclei so obtained are enumerated in a hemocytometer. At intervals throughout the experiment, the nuclei are freed from the cells of various cultures comprising the series and are counted in the same manner. Fluid renewals are accomplished regularly by centrifuging down the loose cells and withdrawing and replacing measured quantities of the cell-free medium. These procedures, which had been worked out with great care, were designed specifically for free wandering cells (e.g., Earle's L strain mouse cells) supplied with media containing blood serum, tissue extracts, and other naturally-occurring ingredients. But it was soon found that the procedures had to be modified considerably for cells cultivated in synthetic media. It is the purpose of the present communication to report the revised procedures and to demonstrate their usefulness in the present undertaking.

Modified Procedures for the Preparation of Replicate Cell Cultures

A brief description will now be given of the various items of special equipment used in preparing the cultures. Detailed specifications of the more elaborate items have already been published by Earle and his associates (4).

(1) Earle's T-15 culture flasks (14). These flasks* are similar to the T-12 flasks, but of improved design. They are used with 2.5 ml. of medium. Wooden racks hold the flasks upright in groups of eight during their preparation and during subsequent fluid renewals, and glass caps are used to close them temporarily during these operations. The usual gassing equipment is required (8% CO_2, 21% O_2, 71% N_2) for adjusting the pH of the medium before closing the cultures with rubber stoppers and setting them away for incubation.

(2) Kolle (K) culture flasks† are used in this laboratory instead of Earle's T-60 flasks for the propagation of large cell populations from which washed

* *Kontes Glass Co., Vineland, N.J.*
† *Corning Glass Works, Corning, N.Y.*

cell suspensions are prepared for the quantitative experiments made in T-15 flasks. K flasks have a diameter of 12 cm. and are used with 20 ml. of medium. The flasks are supplied with large rectangular necks that are converted by a local glassblower to round necks accommodating No. 6 rubber stoppers. Care is taken to eliminate flasks having too great a convexity of the inner bottom surface.

(3) Steel rakes, 13 in. in length, for scraping cells from K flasks. The blade, made from the square end of a 9-mm. wide stainless steel spatula, is fitted to a steel handle with a plastic hand-grip; 5 mm. back from the tip, the blade is bent at 90° to provide the scraper.

(4) Earle's sieve unit for removing any clumps that may be present in the cell suspension, which is passed through two platinum alloy screens, one 80-mesh, the other 150-mesh. (The screens used in this laboratory are made from an alloy containing 10% rhodium.)

(5) Earle's reservoir and stirrer assembly, consisting of a motor-driven glass stirrer with a screw-type blade that rotates in a cylindrical reservoir designed to hold the cell suspension (80 ml.) while it is being stirred and delivered as replicate samples into the T flasks. Near the top of the reservoir, there is an inlet for gassing the cell suspension during the continuous stirring. The reservoir has a broad base that is closely joined to a special 3-way stopcock with a gas inlet and a delivery tip calibrated to dispense 0.5 ml. of the cell suspension. After the delivery tip has been charged with cell suspension from the reservoir, the sample is expelled into a T flask by means of a gas mixture introduced through the gas inlet of the stopcock. As the delivery tip is charged with cell suspension from the reservoir, some of the suspension is lost from the tip; this excess is caught in a beaker and, after all the T flasks have been seeded, it may, in an emergency, be used for other purposes. For protection against falling dust particles, the top of the reservoir is provided with a loose cap which, in turn, is surmounted by a glass sleeve held by rubber tubing to the stem of the stirrer. The delivery tip is protected by a glass bell held in place with a cork.

(6) Stirring motor, Sunbeam Mixmaster.

(7) Earle's flask-centering frame and pipette-positioning clamp and shield for withdrawing measured volumes of supernatant from the T flasks when the medium is to be replenished.

Although experiments have been made with several cell strains, this report deals only with assays made with Earle's L strain cells, which were derived in 1940 from adult mouse connective tissue and continued since 1948 from the progeny of a single cell (13). Cell populations for the preparation of the washed cell suspensions are cultivated for seven days in modified Kolle (K) flasks in 20 ml. of medium consisting of 40% horse serum (from selected horses yielding serum that is nontoxic for murine cells), 3% chick embryo extract (prepared in a motor driven pyrex homogenizer from 12-day-old embryos), 57% Earle's balanced salt solution (3), and, a final concentration

of 0.002% phenol red. The K flasks are seeded with 2–3 ml. of the washed cell suspension left in the reservoir after the T flasks have been prepared, but for this purpose the washed suspension is drained from the reservoir and centrifuged for 5 min. at 1100 r.p.m. Then, after the great bulk of the cell-free supernatant has been removed and discarded, the cells are redistributed in the remainder and divided between four fresh K flasks. These K flasks will furnish the cell suspension for the replicate culture experiment to be made one week later. No effort is made to determine the number of cells in the washed suspension until after the new series of T flasks have been prepared. An effort is made, however, to estimate roughly, by visual inspection, the appropriate number of K flasks required to give a suspension yielding not more than 300,000 cells per culture. Ordinarily, a good K flask will yield 40–50 million cells. Cellophane is not used on the floor of the flask to provide additional growing surfaces, but a small fragment of it, about 2 sq. in., is sterilized in the flask and is used when the flask is harvested, in conjunction with the steel rake, as a convenient mop with which to free the cells from the glass. When the K flask is prepared, the cellophane is moistened with medium and placed on the roof of the flask, where it remains until the time of harvesting. On the fourth day of incubation, one half of the medium in each K flask is renewed.

The cells for the inoculum are harvested from three to four K flasks, transferred together with their medium to a 250-ml. centrifuge bottle, and centrifuged for 10 min. at 1100 r.p.m. The supernatant is discarded and replaced by Earle's balanced salt mixture; and the cells are redistributed in the saline by means of a fine hand-drawn pipette. After another centrifugation, the supernatant is again discarded and the cells are resuspended in the final volume (80 ml.) of balanced saline. The suspension is then passed through the platinum screens of the sieve unit and directly from the sieve unit into the reservoir chamber of the stirring device. It has been found that 80 ml. is the least amount of suspension required for the utmost uniformity in the replicate cultures that are prepared and in the samples that are taken for other purposes. The suspension is stirred for 15 min. before it is dispensed into the series of T flasks, in the earlier experiments 48 in number, more recently 20.*

During the preparation of the T flasks, samples with double the amount of inoculum are taken to determine the average cell population introduced into each culture. Thus, after a few trial samples have been discarded, the first of three double samples is taken, in a flask without medium, to be counted eventually by enumerating the liberated, stained nuclei in a hemocytometer

** The relative uniformity of the cell population present in the 0.5-ml. samples dispensed by the reservoir and stirrer assembly has also been tested by making DNAP determinations (6) on a series of 50 theoretically uniform samples drawn from the apparatus most frequently used. Because the first 24 samples were more uniform than those comprising the second half of the series, it was concluded (6) that smaller sets of from 20 to 25 cultures provided more uniform inocula at the outset and therefore contributed less to the final variations in the replicate series.*

TABLE I

STAINED NUCLEI COUNTED IN REPRESENTATIVE SAMPLES OF A WASHED-CELL SUSPENSION (STRAIN L) USED AS THE INITIAL INOCULUM FOR 20 REPLICATE CULTURES

Double inoculum sample (1.0 ml.)	Nuclei counted in 5.4 mm.^{3} of diluted suspension*	Operator making counts	Nuclei per single sample (0.5 ml.)
I	114, 120	A	258,000
	122, 130	B	
	111, 100	C	
II	98, 119	A	253,000
	114, 120	B	
	113, 120	C	
III	105, 103	A	268,000
	131, 131	B	
	115, 139	C	

* *Each pair of numbers represents nuclei counted by one individual in 1.8 mm.^{3} (all nine squares of each of two hemocytometer chambers) of diluted suspension of stained nuclei.*

(Table I). The second sample is taken midway through the series of T flasks, and the third sample is taken at the end of the series. In many experiments, another set of three double samples is taken in test tubes at the beginning, midway through the series, and at the end, for the estimation of the original cell population by desoxyribonucleic acid phosphorus (DNAP) determinations (6).

The flasks are not inoculated with the washed cell suspension until after the experimental solutions have been placed in all of them. Ordinarily, each experiment is designed to test two solutions, one against the other (Table II). The flasks are numbered consecutively in the order in which the cell inoculum is to be added and are placed in separate sets of racks, with the odd numbered flasks intended for the first test solution in one set, and the even numbered flasks intended for the second test solution in the other set. This procedure ensures that each solution under test will be inoculated with cells that have been stirred for approximately the same length of time in the same volume of fluid.

The rubber stoppers used to close the culture flasks in these experiments were identical, presumably, with those tested in this laboratory a few years ago (11) and found to be relatively nontoxic for roller-tube cultures of chick embryo tissues in synthetic medium No. 199. Over the past few months, however, recent shipments of these stoppers have been tested again with synthetic media, by a different method. This time, the stoppers to be tested were cut into small fragments, 30–40 of which were autoclaved in each empty culture flask. Cultures were then prepared in these flasks by adding synthetic medium and washed cell suspensions of Earle's L strain. Under these conditions, the stoppers previously tested killed the cells within 24 hr., whereas a sample stopper of a new formulation (silicone) proved to be virtually nontoxic,

TABLE II

STAINED NUCLEI COUNTED IN SEVEN-DAY-OLD REPLICATE CULTURES BELONGING TO A SERIES OF 20, EACH INOCULATED ORIGINALLY WITH A WASHED SUSPENSION AVERAGING 260,000 CELLS PER CULTURE (SEE TABLE I)

Replicate culture No.	Nuclei counted in 5.4 mm.³ of diluted suspension*	Operator making counts	Nuclei per culture	Synthetic medium No.†
18718— 1	231, 231	A	956,000	635
	190, 197	B		
	253, 188	C		
2	242, 288	A	1,160,000	703
	277, 270	B		
	240, 249	D		
7	211, 179	A	900,000	635
	218, 223	B		
	184, 200	C		
8	324, 330	A	1,250,000	703
	248, 233	C		
	270, 282	D		
13	181, 188	A	920,000	635
	236, 246	B		
	194, 197	D		
14	336, 306	A	1,367,000	703
	310, 306	B		
	281, 307	D		
19	216, 192	A	955,000	635
	243, 236	B		
	225, 177	D		
20	277, 245	A	1,154,000	703
	271, 259	B		
	246, 260	D		

** Each pair of numbers represents nuclei counted by one individual in 1.8 mm.³ (all nine squares of each of two hemocytometer chambers) of diluted suspension of stained nuclei.*

† Synthetic media 635 and 703 are described in a subsequent communication (7).

and the cells continued to multiply on the glass floor of the flask beneath dozens of floating fragments of the autoclaved stopper. Unfortunately, it has only recently been possible to obtain the silicone stoppers in the sizes required.‡

Modified Procedures for the Care of Replicate Cell Cultures

In this laboratory, the medium placed on replicate T-flask cultures is renewed every four days (or twice a week) by withdrawing 1.0 ml. of the fluid from each flask and replacing it with fresh medium. According to the procedures of Earle and his associates (4), the fluid withdrawals are accomplished by centrifuging the flasks at 300 r.p.m. for 20 min., after which the supernatant fluid is withdrawn by means of 1-ml. volumetric pipettes operated by a syringe barrel and piston in a special flask- and pipette-positioning

‡ *The West Co., Phoenixville, Pa.*

assembly. These procedures, which make it possible to remove part of the medium without removing any loose cells that may be present, have been shown by Earle and his associates to be entirely adequate for cells cultivated on glass in media containing blood serum and tissue extracts. In this laboratory, however, it has been found that flasks containing cells cultivated on glass in synthetic media cannot be centrifuged, even at the low speed of 300 r.p.m., without killing a large part of the cell population. During centrifugation, the fluid is gradually drained from the cells at the top, or neck, end of the flask and this displaced fluid serves to protect the cells located at lower levels so long as it continues to flow over them. The final result is that all of the cells in the upper part of the flask are killed immediately, whereas those in the lower part remain alive; and, there is a remarkably sharp line of demarcation between the two zones, the relative areas of which depend on the speed of centrifugation and its duration. To avoid these harmful effects of centrifugation, the fluid withdrawals are now made after the cultures have been allowed to stand vertically in flask racks for 15 min., during which time any loose cells settle into the constricted bottom ends of the flasks. Before the flasks are placed in the racks, the tight-fitting rubber stoppers are replaced by loose-fitting glass caps that may be removed, without agitating the flasks, when the measured quantities of fluid are to be withdrawn and replaced by fresh medium.

Modified Procedures for the Isolation and Enumeration of Nuclei

The procedures for the isolation of nuclei as adapted by Earle and his associates (12) from the work of Marshak (8) and Dounce (2) were found to be completely satisfactory for cells cultivated in media containing naturally-occurring ingredients (blood serum, tissue extracts, etc.) but not for cells cultivated in chemically-defined (synthetic) media. In many experiments, it was found impossible to detach a large proportion of the nuclei from the glass surfaces despite rigid adherence to the prescribed manner of treatment, which consisted essentially of shaking the cultures vigorously during and after one hour's incubation at 37° C. with 2% citric acid. The early attempts to overcome this difficulty were unsuccessful. In some experiments, the period of incubation with citric acid was prolonged; in others, the cultures were agitated continuously during the citric acid treatment. Tests were also made with other alipathic acids having approximately the same pK_1 as citric acid. Of these, strong ascorbic acid ($pK_1 = 4.17$) released the nuclei from the glass quite satisfactorily, as did 30% citric acid, but both of these treatments seriously damaged many of the nuclei. Finally, it was found that a brief pretreatment with 1% tannic acid coagulated the nuclei just enough to prevent gross distortion and to allow the cytoplasm to be stripped from them by subsequent treatment with 30% citric acid; simultaneously, the nuclei were detached from the glass. In addition to the modifications that have been made in the manner of treating the cells for the isolation of nuclei, the procedures of Earle and his associates have been considerably shortened

by eliminating the period of incubation with citric acid and by eliminating the final centrifugation of the isolated, stained nuclei. The revised procedures will now be described in detail.

A. Preparation of Nuclei from Replicate Cell Cultures

(1) Five milliliters of 30% citric acid* are added to each of as many 25-ml., round-bottom test tubes ($\frac{3}{4} \times 6$ in., graduated in 0.5 ml.) as there are cultures to be handled.

(2) Five milliliters of 1% tannic acid are added to each culture flask, which is then restoppered and allowed to lie flat (with the fluid covering the cells) for not longer than two minutes. This maximum period will determine the number of flasks that can be handled simultaneously.

(3) The contents of the first flask are poured into one of the 25-ml. tubes and shaken. Immediately thereafter, 5 ml. of 30% citric acid are placed in the drained flask, which is shaken vigorously and allowed to lie flat (with the fluid covering the cells) for 5–10 min.

(4) The contents of the flask are then added to the 25-ml. tube, after which the flask is again shaken vigorously with 5 ml. of 30% citric acid and the contents added to the tube, which should now contain about 23 ml. (The measurements need only be approximate up to this point, hereafter, they must be exact.)

(5) The tubes are stoppered, shaken, and centrifuged for 20 min. at 1100 r.p.m. (No. 2 International).

(6) After centrifugation, all but 1.0 ml. of the supernatant fluid is removed from each tube by mild suction, care being taken not to disturb the cells.

(7) To each tube is added 1.0 ml. of 0.1% crystal violet, after which the tube is shaken and the contents poured into a 20-ml. beaker.

(8) Thirty per cent citric acid (1.0 ml.) is added to the drained tube, shaken, and poured into the beaker; this is repeated to give a final volume of 4.0 ml. in the beaker. (If a higher dilution is desired, the final volume is increased.)

(9) The contents of the beaker are transferred immediately to a special cell-counting pipette by applying suction to the latter from a mouth tube while the beaker is being agitated by a gentle swirling motion.

B. Preparation of Nuclei from Replicate Samples of Inoculum Used to Seed the Culture Flasks

At the time the cultures comprising a single experiment are prepared from the washed-cell suspension, three flasks, without medium, are inoculated with double samples of the stirred suspension, as follows: (1) at the beginning of the culture series; (2) midway through the series; and (3) at the end of the series. The nuclei isolated from these cells are stained and enumerated as a means of estimating the size of the culture inoculum (see Table I). Because the

** All stock solutions employed in the preparation of nuclei for counting contain 0.01% propionic acid as an antimycotic.*

three samples are not incubated with synthetic media, the original procedures devised by Earle and his associates could be used without modification. In this laboratory, however, the samples are prepared for counting by a shortened procedure that is quite similar to the one just described for the cultures. It is, as follows:

(1) Five milliliters of 2% citric acid are added to each of the three flasks, which are then shaken and allowed to lie flat for 5–10 min.

(2) The contents of each flask are poured into a 25-ml. graduated tube.

(3) Each of the flasks is rinsed with 5 ml. of 2% citric acid, and the rinsings are added immediately to their respective tubes.

(4) The graduated tubes are stoppered, shaken, and centrifuged for 20 min. at 1100 r.p.m.

(5) After centrifugation, all but 1.0 ml. of the supernatant is removed from each tube by suction.

(6) To each tube is added 1.0 ml. of 0.1% crystal violet, after which the tube is shaken and the contents poured into a 20-ml. beaker.

(7) Two per cent citric acid (1.0 ml.) is added to the drained tube, shaken, and poured into the beaker; this procedure is then repeated to give a final volume of 4 ml. in the beaker.

(8) The contents of the beaker are transferred immediately to a cell-counting pipette, in the usual manner.

The special counting pipettes (12) must be large enough to hold at least 4 ml. of suspension; for heavier suspensions, larger pipettes are used in order to accommodate higher dilutions. If the counting is to be delayed, the counting pipettes are stored at 4° C. in sealing devices consisting of two brass cups fitted with rubber cushions and connected by wire springs. At the time of counting, the pipettes are clamped in Bryan-Garrey blood-cell-counting rotors and rotated for at least 15 min. to establish and maintain a uniform dispersion of nuclei before samples are taken. Before the hemocytometer is charged, the contents of the tip of the pipette are discarded. Ordinarily, each suspension of nuclei is sampled and counted by three individuals each of whom counts the nuclei in two hemocytometer chambers, each chamber consisting of nine 1-mm. squares (see Tables I and II). Thus, if 1290 nuclei are counted in a total of 54 squares, a volume of 5.4 $mm.^3$ is counted from each suspension. If the suspension had been diluted to 4000 $mm.^3$, the number of nuclei per culture would be calculated as follows: $1290 \times 4000/5.4 = 956{,}000$.

According to Earle and his associates (12), 750–800 nuclei must be counted in order to attain an accuracy of 90%, 95% of the time. If this number is not achieved by counting the nuclei in six hemocytometer chambers (5.4 $mm.^3$), more chambers are usually counted. For assays of synthetic media, the ideal experiment would appear to be one in which the washed-cell inoculum averages 200,000 to 300,000 cells per culture.

In selecting a group of cultures for nuclear counts, and for DNAP determinations (6), an effort was always made to choose pairs of cultures that had received their cell inoculum in sequence. An effort was also made to choose pairs that were equally separated from one another in the series (see Table II). Thus, cultures 1, 7, 13, and 19, in solution A, would be expected to compare favorably with cultures 2, 8, 14, and 20, in solution B. The reasons for this manner of selection will be presented in a subsequent communication (6).

Preliminary, Nonquantitative Screening Tests with Washed Cell Suspensions

At least once a week, several synthetic solutions are tested in a preliminary manner by means of a greatly simplified version of the quantitative procedures already described. These tests, which are usually made in Carrel flasks, are designed primarily to compare the effects of various levels of a substance of nutritional interest and to eliminate all toxic combinations of substances before testing them by the more elaborate replicate culture procedures. This method is also used to advantage as a quick and simple means of testing basic media made from freshly prepared stock solutions or from stock solutions made from new lots of chemicals not previously tested. Except for a few culture flasks, of almost any design, the procedure requires no special equipment and often gives as much information as the more elaborate quantitative procedures. Ordinarily, the tests are made from the same washed-cell suspensions used in the preparation of the replicate cultures. The washed-cell suspension is agitated by hand, rather than mechanically, and an ungraduated, hand-drawn pipette is used to transfer one or two drops of the agitated suspension to each of the culture flasks. The effects of the various solutions are judged, not by isolating and counting the cell nuclei, but by examination of the cultures at intervals under the microscope. Some experiments have to be kept for as long as 20–30 days, or more, before it is possible to detect significant differences between cultures in different solutions. Three cultures are prepared for each solution tested, and all culture fluids are renewed twice a week. No precautions are taken to guard against the loss of unattached cells. The method can also be used to advantage in testing the relative toxic or growth-promoting properties of substances derived from, or added to, natural media.

Discussion

During the past year, Earle and his associates (5) reported the results of three experiments in which chemically-defined (synthetic) media were compared with media containing horse serum and embryo extract. In the first experiment, the synthetic medium chosen was Morgan, Morton, and Parker's solution 199 (9) prepared at the National Cancer Institute, Bethesda, Md. In the second experiment, three synthetic media were used: solution 199 prepared in Bethesda; solution 199 prepared in our Toronto laboratory; and, White's solution 3 (15). In the third experiment, only White's medium was

used. There was no indication in the report of the number of cultures studied in the first and third experiments, but the results of the second experiment were based on four cultures in each medium counted on the sixth day and another set of four each counted on the 11th day. The results showed that the number of surviving cells counted in cultures treated with solution 199 prepared in Bethesda had fallen at the end of six days to 65% of those present in the original inoculum, and to 31% at the end of 11 days. Also, cells treated with solution 199 sent from Toronto survived for shorter periods than those in solution 199 prepared in Bethesda; and cells treated with White's medium fared even worse. According to the authors, these studies were "concerned with determining quantitatively whether, for the cell strain used under the prescribed experimental conditions, the chemically defined media studied could be considered as basal nutritional media adequate to maintain cell populations at or close to the given initial inoculum level." Because of the results obtained, the authors concluded that neither of the chemically defined media studied could be considered as a basal nutritional medium for the cell strain used.

The cells used in the investigation of Earle and his associates, and in the present one, were from the same source, namely, Earle's L strain derived in 1940 from adult mouse connective tissue and continued since 1948 from the progeny of a single cell (13). Also, the experimental procedures devised by Earle and his associates for the preparation of replicate cultures from these cells were followed in every essential detail in the present investigation. But it was soon found that it was not possible to follow Earle's original procedures in their entirety if the cells were to be cultivated in synthetic media. Washed cell suspensions placed for cultivation in synthetic medium 199 would, in many instances, fail to adhere to the glass, or, if they did so, the vast majority of them would not remain adherent for more than a few days. By the seventh day, the cultures usually showed great variability, not only in the density of the cell population and in the general appearance of the cells, but also in the number of cells that remained on the glass.

It is not feasible, nor would it be particularly informative, to record the large number of experiments that were made with a wide variety of synthetic media in an effort to overcome these difficulties. Gradually, however, the cultures improved and promising results began to be achieved when their cell populations were measured by making nuclear counts and DNAP determinations (6), on the 7th and 12th days of incubation. This gradual improvement was due partly to beneficial changes that were made in the composition of the synthetic media and partly, no doubt, to our increased facility in handling the quantitative culture techniques. Although the culture techniques are not difficult to handle, they are exacting, and many factors quite unrelated to the medium can determine the success or failure of a particular experiment. Among these contributing factors may be listed the following: the condition, at the time of harvesting, of the cells providing the inoculum; the manner in

which the washed cell suspensions are prepared; the peculiarities of the various reservoir and stirring assemblies that are used; the volume of the cell suspension in relation to the capacity of the reservoir, the length of the stirrer, and the number of cultures to be prepared; the cleanliness of the glassware and the composition of the rubber stoppers used to close the culture flasks; and, the facility with which all the various operations are accomplished.

As the appearance of the cultures improved and as more cells remained adherent to the glass floor of the flasks, new difficulties were encountered that were quite as frustrating as those just described. Although there was ample visual evidence of cell multiplication, there was little correlation between the appearance of the cultures under the microscope and the results of the nuclear counts. It was then discovered that a large proportion of the nuclei were not being counted but remained adherent to the glass and were left behind in the flasks after the cultures were treated with citric acid and crystal violet. The measures that were taken in an effort to correct this situation have been described (p. 312). Almost simultaneously, however, a new source of error was found in cells that were being killed by centrifugation at the time the culture fluids were renewed. This source of error was easier to eradicate, for if the cultures were left standing in a vertical position for 15 min., it was possible to remove part of the fluid without also removing any cells that were in suspension.

It should be stated that none of the difficulties described has been encountered in applying Earle's original replicate culture techniques to cells propagated in media containing such naturally-occurring ingredients as blood serum and tissue extracts. It is possible that the presence of these substances makes for physicochemical conditions in the cultures that are quite different from those existing when the media are entirely synthetic. In any event, cultures containing serum and tissue extracts can be centrifuged at moderate speeds without killing the cells in the upper portions of the flasks, and cells cultivated in media containing these ingredients readily release their nuclei when treated with 2% citric acid. It would seem, therefore, that the experience of Earle and his associates in their two experiments with solution 199 may have resulted partly from their failure to realize that cells propagated in chemically-defined media must be handled quite differently from those propagated in media containing naturally-occurring ingredients. It should also be pointed out that the various sources of error that have been found in applying their original procedures to replicate cultures in synthetic media might never have been discovered were it not for the fact that the synthetic media were undergoing continuous improvement in this laboratory during the time the replicate culture techniques were being introduced. Although solution 199 has now been superseded (7), it did serve to excellent advantage as a basal medium for testing the nutritional qualities of a wide variety of substances.

Acknowledgments

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NUTRITION OF ANIMAL CELLS IN TISSUE CULTURE

VIII. DESOXYRIBONUCLEIC ACID PHOSPHORUS AS A MEASURE OF CELL MULTIPLICATION IN REPLICATE CULTURES[1]

By George M. Healy, Dorothy C. Fisher, and Raymond C. Parker

Abstract

A chemical method of estimating the size of cell populations in replicate cultures has been devised for use with certain cell strains from the mouse and rat. The method is based upon the determination of desoxyribonucleic acid phosphorus (DNAP). Although the method may be used as an alternative to the procedure of counting isolated, stained nuclei in a hemocytometer, it actually supplements such measurements and provides useful information on the DNA-content per cell. The method consists of a modified Schmidt and Thannhauser separation of ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) followed by the spectrophotometric estimation of the orthophosphate. The present communication describes the procedures in detail and reports recoveries of RNAP and DNAP from mixtures of highly polymerized nucleic acids and various types of biological material. Good correlation was found between nuclear counts and DNAP values in natural media (containing horse serum and chick embryo extract) and in synthetic (chemically-defined) media. Finally, the efficiency of the dispensing apparatus most frequently employed in preparing replicate cultures from continuously stirred, washed cell suspensions has been determined by measuring the DNAP content of serially dispensed samples.

Introduction

A method of measuring animal cell multiplication in cultures by determining the amount of nucleoprotein phosphorus present in the tissues was introduced by Willmer (23), who employed the chemical procedure of Berenblum, Chain, and Heatley (1). Willmer selected this means of estimating growth on the assumption that nucleoprotein is the most characteristic constituent of the cell nucleus, and found good agreement between nucleoprotein phosphorus and the dry weights of cultures as reported by Laser (14). Cunningham and Kirk (3) also considered nucleoprotein phosphorus measurements to be a satisfactory means of estimating growth. It is now known that estimations of cell populations based on the total nucleoprotein phosphorus are not too reliable inasmuch as the ribonucleic acid (RNA) portion of the nucleoprotein varies with the physiological state of the cells (2). Fortunately, however, the quantity of desoxyribonucleic acid (DNA) per cell is reasonably uniform for each species (4); and, the germ cells contain half the amount of DNA found in the somatic cells (16). In 1945, Schmidt and Thannhauser (21) and Schneider (22) reported methods for the quantitative determination of RNA and DNA in animal tissues. The Schmidt–Thannhauser procedure was based on the different behavior of these compounds during mild treatment with alkali, whereas Schneider's method involved the colorimetric determination of RNA and DNA in separate portions of a trichloroacetic acid extract of the

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tissues. In 1949, Davidson, Leslie, and Waymouth (5) employed a modified Schmidt–Thannhauser procedure to follow changes in RNA and DNA in fibroblast cultures by making phosphorus (P) determinations in these fractions. In our laboratory, a slight variation of this modified procedure was chosen for the estimation of RNAP and DNAP in replicate cell cultures, and advantage was taken of a highly sensitive colorimetric method that has been developed more recently (12) for the determination of P. It is the purpose of the present report to describe the method and to demonstrate: (1) that DNAP values serve as a reliable index of the size of cell populations cultivated either in natural or in synthetic media, and (2) that DNAP values can be used to advantage in testing the efficiency of replicate culture procedures.

Materials and Methods

Cell Strain

The cells used in this investigation were derived from Earle's L strain that was isolated in 1940 from the subcutaneous tissue of an adult mouse and continued since 1948 from the progeny of a single cell (19).

Culture Media

The media were of two types, *natural* (containing naturally-occurring ingredients) and *synthetic* (chemically-defined). The only natural medium used was one consisting of 40% horse serum (from selected animals yielding serum nontoxic for murine cells), 3% chick embryo extract prepared in a motor-driven pyrex homogenizer from 12-day-old embryos, and 0.002% phenol red, in a balanced salt solution containing glucose (8). The synthetic media were modifications of solution 199, which was developed in this laboratory some years ago (17).

Replicate Cell Cultures

The procedures followed in the preparation of replicate cell cultures were originally developed by Earle and his associates (9) and adapted by us for use with synthetic media. In brief, washed cell suspensions were prepared from cultures of Earle's L strain grown for seven days in 20 ml. of natural medium in large Kolle flasks. After the washed cell suspensions were screened to eliminate cell clumps, they were transferred to a special reservoir chamber in which they were stirred mechanically and dispensed, eventually, as replicate 0.5-ml. samples into a series of 20 or 48 T-15 culture flasks to which the medium had already been added. In experiments in which two media were to be compared, each was placed in alternate flasks. The number of cells placed in each culture of the series was estimated by counting the cell nuclei in representative samples of the stirred inoculum. The samples were prepared for counting by treating the cells with tannic and citric acids followed by low speed centrifugation and light staining with crystal violet. The liberated, stained nuclei so obtained were counted in a hemocytometer. At intervals throughout the experiment, the nuclei were freed from the cells of various

cultures comprising the series and counted in the same manner. Twice a week, fluid renewals were accomplished by allowing any loose cells to settle and by withdrawing and replacing measured quantities of the cell-free medium. Other samples of the cell inoculum and other cultures from the series of T-flasks were withdrawn for DNAP determinations, according to the procedures to be described. Cultures selected for DNAP determinations were sometimes frozen until a convenient number had accumulated, or until an experiment had been terminated.

RNAP and DNAP Determinations

The Schmidt–Thannhauser procedure for the separation of RNA and DNA (21), as modified by Davidson, Leslie, and Waymouth (5), was further modified in the present investigation. One milliliter of 0.01% crystalline insulin was added prior to each trichloroacetic acid precipitation to ensure completeness of the precipitation (20), and MacFadyen's reagent (15) was used instead of 10% trichloroacetic acid for the initial acid extraction of nucleoprotein. Following the lipid extractions and mild alkaline hydrolysis (*N* NaOH, 37° C., 12 hr.), the separated RNA and DNA were oxidized with 0.4 ml. of mixed (1 : 2) reagent grade sulphuric–perchloric acids. The orthophosphate obtained from these fractions was measured by the method of Griswold, Humoller, and McIntyre (12). The molybdenum blue color was developed in a final volume of 4 ml., and its extinction was determined in a Hilger Uvispeck spectrophotometer at 830 mμ, the wave length of maximum absorption, against a reference blank that was carried through the entire procedure. Under these conditions, the standard curve relating optical density to micrograms of P was linear from 0 to 2.5 micrograms of P. It should be noted, however, that 4 ml. of solution will not fill a 4-cm. absorption cell, the capacity of which is 10 ml. But the absorption cells can be raised by means of small spacers inserted in the cell carriage, thus enabling the collimated beam of light transmitted through the limited aperture of the Hilger instrument to pass through the solution. If smaller absorption cells are used, the standard curve is linear over a wider range of P with a consequent loss of sensitivity for submicrogram quantities.

Reagents for the P determinations were selected for their low P content. Organic solvents used for the lipid extractions were redistilled in an all-pyrex apparatus. The reducing agent, 1-amino-2-naphthol-4-sulphonic acid, was purified by the method of Fiske and Subbarow (10). All water that was used was passed through a Barnstead still and subsequently through a mixed bed ion-exchange column (13). All glassware, including the T-15 culture flasks, was cleaned by heating to 90° C. in 5 *N* reagent grade nitric acid and, after cooling, was rinsed in tap water, then in distilled water and finally in distilled ion-exchange water. Microtissue grinders,* calibrated with a line at 4 ml., were found to be convenient vessels in which to conduct the entire chemical procedure, without loss due to transfer.

* *Arthur H. Thomas Co., Philadelphia, Pa.*

Results

Recovery of Known Amounts of RNA and DNA Added to a Liver Homogenate

Total P determinations, in duplicate, were made on 1-ml. samples of 0.01% stock solutions of pure, undegraded RNA and DNA, both separately and together. P determinations were also made on like amounts of RNA and DNA that had been combined, separately and together, with 0.5-ml. portions of an extract of embryo chick liver homogenate; and other determinations were made on the liver homogenate alone (Table I). The recoveries of RNA and DNA were quite satisfactory, and it is believed that this was due in large measure to the use of MacFadyen's reagent and aqueous insulin, as already described. The method was further tested by making DNAP determinations on samples of a washed suspension of fowl erythrocytes, and hemocytometer counts were made on comparable samples of the suspension. The DNAP value obtained, 2×10^{-7} μgm. per cell, was in good agreement with the values obtained by others (6).

TABLE I

RECOVERY OF KNOWN AMOUNTS OF RNA AND DNA ADDED TO AN EXTRACT OF CHICK LIVER HOMOGENATE

Samples* (in duplicate)	Treatment	P (μgm.)	Recovery (%)
RNA	Oxidized	8.7	—
DNA	"	7.6	—
RNA + DNA	"	16.2	99.5
RNA + LH	S & T†	12.9	93
DNA + LH	"	12.0	96
RNA + DNA + LH	"	21.2	101
LH	"	4.8	—

* *RNA and DNA: 0.01% solutions (1.0 ml.).*
LH: liver homogenate, diluted (0.5 ml.).
† *Modified Schmidt and Thannhauser (21) procedure.*

Correlation Between Nuclear Counts and DNAP Values for L Strain Mouse Cells

Quantitative experiments were made to compare the growth-promoting properties of natural and synthetic media. In these experiments, DNAP determinations and nuclear counts were made concurrently on cultures from various replicate series that ranged in population from 200,000 cells per culture in one series to 3,000,000 cells per culture in another. Eighteen representative nuclear counts and their corresponding DNAP values are presented graphically in Fig. 1. Each point represents the average values of DNAP determinations made on at least two cultures of a replicate series and nuclear counts made on at least two other cultures of the same series, cultivated in the same medium.

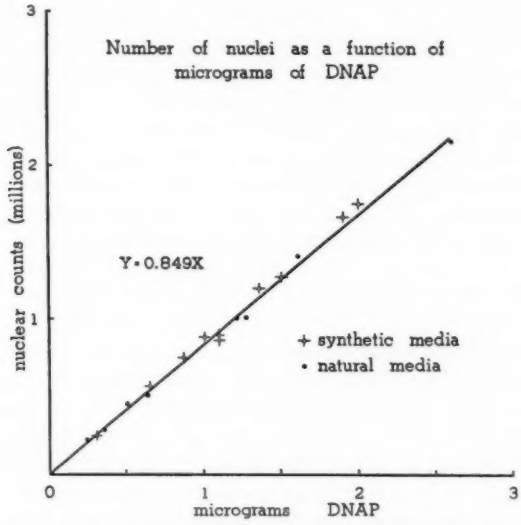

FIG. 1. The relationship between nuclear counts and DNAP values. The straight line, $y = 0.849x$, was determined by the method of least squares.

A statistical analysis of the relationship existing between the DNAP values and the nuclear counts was carried out on the assumption that the relationship was of the form $y = ax^b$, where y = DNAP and x = number of cell nuclei. The DNAP value was made the independent variable because the data indicated a larger variability in the nuclear counts than in the chemical determinations. Because the variability was greater for the higher cell populations, logarithms of the individual counts were used and the relationship was expressed in logarithmic form. The relationship was then estimated for the two types of media separately. From the constants derived (Table II), it is apparent that the relationship between the nuclear counts and the DNAP

TABLE II

CONSTANTS OBTAINED FOR THE RELATIONSHIP, $y = ax^b$, BETWEEN DNAP VALUES AND NUCLEAR COUNTS IN REPLICATE CULTURES OF L STRAIN MOUSE CELLS

	Value obtained for constants	
	Log a	b
Cultures in synthetic media	-0.072 ± 0.010	1.033 ± 0.042
Cultures in natural* media	-0.071 ± 0.014	0.996 ± 0.038
All cultures	-0.070 ± 0.010	1.012 ± 0.028

** 40% horse serum and 3% chick embryo extract, in a balanced salt solution containing glucose.*

values is the same for the two types of media; the constant b does not differ significantly from 1. The variations of the points about the line could be accounted for by the error inherent in the nuclear counts. It would seem, therefore, that the relationship can be adequately described by the linear equation $y = 0.849x$ over the range of nuclear counts and DNAP values that were studied.

It is also interesting to note that the growth response curves for L strain cells are sigmoidal in shape, regardless of whether the medium in which they are cultivated is natural or synthetic.

Efficiency of the Dispensing Apparatus Used in the Preparation of Replicate Cell Cultures

The close correlation between DNAP values and nuclear counts made it possible to determine the efficiency of the replicate culture procedures. DNAP determinations on a set of serially dispensed, and theoretically uniform, replicate samples were carried out in order to estimate that part of the final error that might be attributed to variations in the number of cells present in the inoculum. For this purpose, an unusually heavy washed suspension of Earle's L strain mouse cells was prepared according to the techniques described in an earlier communication (18). A series of 48 samples (0.5 ml. each) were then drawn from the stirring and dispensing unit, and the DNAP per sample was determined for the entire series.

The first 24 samples were remarkably uniform in cell population, as measured in terms of their DNAP values. The remaining samples of the series, however, showed considerable variation. Also, statistical analysis showed that within sets of three consecutive samples of the series the standard deviation was 2.8% of the mean, whereas it increased to 9.6% of the mean when comparisons were made between different sets of three samples each. It was concluded, therefore, that smaller sets of replicate cultures (e.g., 20 to 25 cultures) provide more uniform populations at the outset and contribute less to the final variations. It was further concluded that in selecting a group of cultures from a series for nuclear counts, and DNAP determinations, an effort should always be made to choose pairs of cultures (if two solutions were being tested), or sets of three (if three solutions were being tested) that had received their cell inoculum in sequence and which included the two (or three) test solutions. This scheme had been followed throughout the work.

Discussion

Many investigators have attempted to estimate growth in tissue cultures either by measuring certain chemical constituents of the cells or by measuring changes occurring in the chemical constitution of the medium. But such procedures were never entirely satisfactory because they were always based on the estimated weight, volume, or surface area of sister cultures that were rarely identical. It has been the purpose of the present report to show that these difficulties may be overcome by combining Earle's replicate culture

procedures with other procedures designed to measure a constituent of the cell nucleus (DNA) that is present in constant amount in almost all somatic cells of a given species. In many experiments in which both natural and synthetic media were used, a remarkably close correlation was observed between nuclear counts and the DNAP content of the cultures. And, from the information presently available, it seems clear that DNA is the most suitable cell constituent for this purpose. Certainly, the RNAP of the cells shows greater fluctuations in amount. Also, when the modified Schmidt and Thannhauser method is used, the DNAP is obtained free of all other phosphorus, whereas the P of the RNA fraction cannot be readily dissociated from the P of phosphoprotein (7). It is interesting to note that Goldberg, Klein, and Klein (11) have made a study of Ehrlich ascitic mouse tumors in which cell populations of known size were analyzed for nucleoprotein P and RNAP, and the DNAP value was then obtained by calculating the difference between the two. Although this indirect method of determining DNAP was the one proposed in the original communication of Schmidt and Thannhauser (21), it is believed that the modified procedure used in the present study, in which DNAP determinations are made directly on the precipitated DNA, results in greater sensitivity and in increased specificity.

It should be stated that although DNAP determinations can be used as a reliable measure of the size of cell populations in replicate cultures, the procedure of making nuclear counts is not being abandoned. DNAP determinations require microchemical manipulations extending over a period of two days, whereas nuclear counts can be made in a few hours. Under certain circumstances, however, the chemical method is definitely to be preferred. Thus, cultures may be withdrawn at regular intervals from the replicate series and kept frozen until the experiment has been terminated, at which time they may all be handled together. It is also possible to withdraw cultures for freezing on days when it would not be convenient to work them up by either method. Moreover, the chemical method can be handled by one person, instead of the three usually required for statistically reliable nuclear counts. For almost a year, in this laboratory, both methods were used concurrently in all replicate culture assay experiments, as a means of comparing the two methods. But now that the two methods can be used interchangeably, either one or the other is chosen, in advance, for each experiment, the choice depending upon the considerations already mentioned.

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NUTRITION OF ANIMAL CELLS IN TISSUE CULTURE

IX. SYNTHETIC MEDIUM No. 703[1]

By George M. Healy, Dorothy C. Fisher, and Raymond C. Parker

Abstract

Previous reports from this laboratory described a synthetic medium, solution 199, that contained 60 ingredients and supported cell life for an average period of four to five weeks. The assays were carried out in roller tubes in which chick embryo mesenchyme tissues were cultivated directly on the glass. More recently, the synthetic media have been improved very considerably, and Earle's replicate culture assay procedures have been adapted for use in testing them. By means of these techniques, it has been possible to make accurate quantitative experiments with new synthetic solutions some of which are capable of yielding, in one week, five- and sixfold increases in the population of replicate cultures prepared from washed suspensions of Earle's L strain mouse cells. This was acommplished by omitting the purines and pyrimidines from solution 199, by adding greatly increased levels of cysteine, ascorbic acid, and glutathione, and by the addition of certain purified coenzymes. In solution 703, which is described in detail, cultures of L strain cells have been maintained in a healthy, active state for over five months, with renewal of the medium twice a week.

Introduction

An earlier report from this laboratory (11) described a synthetic (chemically-defined) medium, solution 199, that consisted of amino acids, vitamins, purines, and pyrimidines, a source of fatty acid (Tween 80), certain intermediary metaboiites and accessory growth factors, and a modified Tyrode's solution containing glucose. This solution supported cell life for an average period of four to five weeks and was used to advantage in hundreds of experiments designed to test additional substances of nutritional interest. In these studies, chick embryo mesenchyme tissues were cultivated on the inner surface of pyrex test tubes, without the use of fibrin clots, and the cultures were treated with a preliminary feeding mixture containing horse serum and chick embryo extract, which was left on the cultures for three to five days while the tissue cells were becoming established. At the end of this time, the medium containing the naturally-occurring ingredients was removed and replaced by synthetic media. The synthetic media were changed three times a week, and the cultures were maintained until living cells were no longer discernible. Eventually, it was found (12) that the tissues could be explanted directly to solution 199 if the latter was allowed to remain on the cultures, without renewal, for a period of five days. It was also found that cultures started directly in the synthetic media survived for approximately the same length of time as cultures started in media containing naturally-occurring ingredients. More recently, reports from other laboratories have indicated that solution 199 has been useful in the propagation of viruses, notably poliomyelitis (4, 16).

[1] *Manuscript received February 2, 1954.*
Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario, with financial assistance from the National Cancer Institute of Canada and the Foster Bequest to the Faculty of Medicine, University of Toronto.

Since the first experiments were reported, the synthetic media have been improved very considerably, and Earle's replicate culture procedures have been adapted for use in testing them (13). By means of these techniques, it has been possible to make accurate quantitative experiments with new basal synthetic solutions that are capable of yielding, in one week, five- and sixfold increases in the population of replicate cultures prepared from washed suspensions of Earle's L strain mouse cells. Also, cultures of L strain cells have been maintained in a healthy, active state in synthetic medium for over five months, with complete fluid changes twice a week. It is the purpose of the present communication to describe the steps that were taken to achieve these improvements in the media and to report the composition of the most adequate one thus far devised, solution 703.

Materials and Methods

Culture Material

Although experiments have been made with several cell strains, this report deals only with assays made with Earle's L strain cells, which were derived in 1940 from adult mouse connective tissue and continued since 1948 from the progeny of a single cell (18). Cell populations for the preparation of the washed cell suspensions used in testing the synthetic media are cultivated for seven days in modified Kolle flasks in 20 ml. of natural medium consisting of 40% horse serum and 3% chick embryo extract, in a balanced salt solution containing glucose. Details of these procedures have already been described (13).

Replicate Cultures Assayed by Nuclear Counts

According to these procedures, which were devised by Earle and his associates (3, 17) and modified in this laboratory for use with synthetic media (13), the number of cells placed in each of a series of T-flask cultures is estimated by counting the cell nuclei in representative samples of a washed and continuously-stirred cell suspension used as inoculum. The samples are prepared for counting by treating the cells with tannic and citric acid followed by low speed centrifugation and light staining with crystal violet. The liberated, stained nuclei so obtained are enumerated in a hemocytometer. After 7 and 12 days' incubation, the nuclei are freed from the cells of representative cultures comprising the replicate series and are counted in the same manner. Fluid renewals are accomplished twice a week by allowing any loose cells to settle to the tip of the culture flasks and by replacing measured quantities of the cell-free medium.

Replicate Cultures Assayed by DNAP Determinations

In a previous communication (7), a method was described for estimating the sizes of cell populations in replicate cultures by making desoxyribonucleic acid phosphorus (DNAP) determinations on the cultures. The method

consists of a modified Schmidt and Thannhauser separation of RNA and DNA followed by the spectrophotometric estimation of the orthophosphate. Because good correlations have been found between nuclear counts and DNAP determinations for cells cultivated both in natural and in synthetic media, the two methods can be used interchangeably, and one or the other is chosen, in advance, for each experiment. In the majority of the experiments reported here, DNAP determinations and nuclear counts were made concurrently on representative T-flask cultures of each replicate series. In experiments designed to test the effect of nucleic acid constituents added to solution 635, the sizes of the original cell inocula and of the cell populations after seven days' cultivation were estimated from DNAP values.

Preliminary Screening Tests with Washed-Cell Suspensions

At least once a week, one or more synthetic solutions are tested in a preliminary manner by means of a greatly simplified version of the quantitative procedures just described. These tests (13), which are usually made in Carrel flasks, are designed to compare the effects of various levels of a substance of nutritional interest and to eliminate all toxic combinations of substances before the solutions are tested by the more elaborate replicate culture procedures. Ordinarily, the tests are made from the same washed-cell suspensions used in the preparation of the replicate T-flask cultures. But the suspension is agitated by hand, rather than mechanically, and an ungraduated pipette is used to transfer one or two drops of the agitated suspension to each of the culture flasks. Also, the effects of the various solutions are judged not by isolating and counting the cell nuclei, or by making DNAP determinations, but by examining the cultures at intervals under the microscope. Three cultures are prepared for each solution tested, and all culture fluids are renewed twice a week. No precautions are taken to guard against the loss of any cells that may be in suspension.

Stock Solutions for the Preparation of Synthetic Medium 703

The components of the synthetic media are obtained commercially* and are employed without further purification. Aqueous stock solutions are prepared with water passed through a Barnstead still and then through a mixed-bed ion-exchange column (8). All stock solutions except Nos. 1 and 9 are stored at 4° C., without filtration, for periods not exceeding 30 days. A fresh lot of solution 1 is made up each time a new batch of medium is prepared; solution 9 is stored in the frozen state until used. The ingredients of synthetic medium 703 are shown in Table 1. The various stock solutions are prepared as follows:

* *Sources of biochemicals: Armour and Co., Chicago, Ill.; Atlas Powder Co., Wilmington, Delaware; British Drug Houses, Ltd., Toronto, Ontario; Distillation Products, Inc., Rochester, N.Y.; Eastman Kodak Co., Rochester, N.Y.; G and W Laboratories, Jersey City, N.J.; General Biochemicals, Inc., Chagrin Falls, Ohio; Hoffman-La Roche, Inc., Nutley, N.J.; Merck & Co., Inc., Rahway, N.J.; Nutritional Biochemicals Corp., Cleveland, Ohio; Pabst Laboratories, Milwaukee, Wis.; Schwarz Laboratories, Inc., New York, N.Y.; and, Sigma Chemical Co., St. Louis, Missouri.*

TABLE I

SYNTHETIC MEDIUM No. 703

	Mgm. per 1000 ml.		Mgm. per 1000 ml.
Inorganic salts		Vitamins (*cont'd*)	
NaCl	6800.0	Folic acid	0.01
KCl	400.0	Choline	0.50
$CaCl_2$	200.0	Inositol	0.05
$MgSO_4 . 7H_2O$	200.0	*p*-Aminobenzoic acid	0.05
$NaH_2PO_4 . H_2O$	140.0	Vitamin A	0.10
$NaHCO_3$	2200.0	Ascorbic acid (vit. C)	50.00
Fe, as $Fe(NO_3)_3$	0.1	Calciferol (vit. D)	0.10
		α-Tocopherol phosphate (vit. E)	0.01
Amino acids		Menadione (vit. K)	0.01
l-Arginine	70.0		
l-Histidine	20.0		
l-Lysine	70.0	Lipid sources	
l-Tyrosine	40.0	Tween 80* (oleic acid)	20.0
dl-Tryptophane	20.0	Cholesterol	0.2
dl-Phenylalanine	50.0		
l-Cystine	20.0		
dl-Methionine	30.0	Coenzymes	
dl-Serine	50.0	Diphosphopyridine nucleotide (DPN)	0.78
dl-Threonine	60.0	Triphosphopyridine nucleotide (TPN)	0.42
dl-Leucine	120.0	Coenzyme A	0.27
dl-Isoleucine	40.0		
dl-Valine	50.0		
dl-Glutamic acid	150.0		
dl-Aspartic acid	60.0		
dl-Alanine	50.0	Miscellaneous	
l-Proline	40.0	Sodium acetate	50.0
l-Hydroxyproline	10.0	Glutathione	10.0
Glycine	50.0	*l*-Glutamine	100.0
Cysteine hydrochloride	260.0	Glucose	1000.0
		Phenol red (pH indicator)	15.0
		Ethyl alcohol (as initial solvent for vits. A, D, and K, and cholesterol)	16.0
Vitamins			
Thiamine	0.01		
Riboflavin	0.01		
Pyridoxine	0.025		
Pyridoxal	0.025	Antibiotics	
Niacin	0.025	Sodium penicillin G (added just before use)	1.0
Niacinamide	0.025	Dihydrostreptomycin sulphate	100.0
Pantothenate	0.01	*n*-Butyl parahydroxybenzoate	0.2
Biotin	0.01		

* *Aqueous Tween 80 also serves as the final diluent of an alcoholic stock solution of vits. A, D, and K, and cholesterol.*

Solution 1. To 400–450 ml. of water stirred continuously and heated to about 80° C. are added the following: phenol red W.S., 15 mgm.; *l*-arginine monohydrochloride, 70 mgm.; *l*-histidine monohydrochloride, 20 mgm.; *l*-lysine monohydrochloride, 70 mgm.; *dl*-tryptophane, 20 mgm.; *dl*-phenylalanine, 50 mgm.; *dl*-methionine, 30 mgm.; *dl*-serine, 50 mgm.; *dl*-threonine, 60 mgm.; *dl*-leucine, 120 mgm.; *dl*-isoleucine, 40 mgm.; *dl*-valine, 50 mgm.; *dl*-glutamic acid monohydrate, 150 mgm.; *dl*-aspartic acid, 60 mgm.; *dl*-α-alanine, 50 mgm.; *l*-proline, 40 mgm.; *l*-hydroxyproline, 10 mgm.; glycine,

50 mgm.; and sodium acetate, 81.5 mgm. After the solution has cooled to room temperature, the following are added: *l*-glutamine, 100 mgm.; cysteine hydrochloride, 260 mgm.; ascorbic acid, 50 mgm.; glutathione, 10 mgm.; and, dihydrostreptomycin sulphate, 100 mgm. The ingredients of Earle's balanced salt solution (18) are then added, as follows: sodium chloride, 6.8 gm.; potassium chloride, 0.4 gm.; calcium chloride, 0.2 gm.; magnesium sulphate ($MgSO_4 \cdot 7H_2O$), 0.2 gm.; sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$), 0.14 gm.; sodium bicarbonate ($NaHCO_3$), 2.2 gm.; and, glucose, 1.0 gm. Finally, the solution is made up to 500 ml. with water.

Solution 2. Two hundred milligrams of *l*-tyrosine and 100 mgm. *l*-cystine are dissolved with moderate heating in 100 ml. of 0.075 *N* hydrochloric acid.

Solution 3. The following B vitamins are dissolved in 200 ml. (final volume) of water: niacin, 25 mgm.; niacinamide, 25 mgm.; pyridoxine hydrochloride, 25 mgm.; pyridoxal hydrochloride, 25 mgm.; thiamine hydrochloride, 10 mgm.; riboflavin, 10 mgm.; calcium pantothenate, 10 mgm.; i-inositol, 50 mgm.; *p*-aminobenzoic acid, 50 mgm.; and choline chloride, 500 mgm. The stock solution consists of a 1 : 50 dilution of this solution with water.

Solution 4. Ten milligrams of *d*-biotin are dissolved in approximately 50 ml. of water containing 1 ml. *N* hydrochloric acid (to increase stability on storage), and the final volume is adjusted to 100 ml. The stock solution consists of a 1 : 100 dilution of this solution with water.

Solution 5. Ten milligrams of folic acid are dissolved in 100 ml. of Earle's balanced salt solution (18).

Solution 6. Two alcoholic tinctures are required to prepare this combined stock solution: cholesterol 10 mgm. per ml. in 95% ethanol; and, menadione (vit. K) 10 mgm. per ml. in 95% ethanol. The following lipid soluble materials are mixed in an aluminum weighing pan: calciferol (vit. D), 10 mgm., dissolved in 1 ml. of the tincture of cholesterol; vitamin A, 10 mgm., dissolved in another 1-ml. portion of the tincture of cholesterol; tincture of menadione (vit. K), 0.1 ml.; and, *n*-butyl parahydroxybenzoate (an antimycotic), reagent grade, 20 mgm. Finally, 10 ml. of a 5% aqueous solution of Tween 80 are added to a 100-ml. volumetric flask together with the alcoholic solutions of vitamins A, D, and K, and cholesterol. The mixture is then made up to a final volume of 100 ml. and warmed to dissolve the cholesterol. The stock solution consists of a 1 : 10 dilution of this solution with water.

Solution 7. Ten milligrams of disodium α-tocopherol phosphate (vit. E) are dissolved in water to make a final volume of 100 ml. The stock solution consists of a 1 : 100 dilution of this solution with water.

Solution 8. Thirty-six milligrams of ferric nitrate, $Fe(NO_3)_3 \cdot 9H_2O$, are dissolved in water to make a final volume of 100 ml. One drop of concentrated nitric acid is added to prevent hydrolysis during storage.

Solution 9. The following partially purified coenzymes are dissolved in a final volume of 100 ml. of water, distributed in 10-ml. portions in pyrex test tubes, and immediately frozen for storage: 12.0 mgm. diphosphopyridine

nucleotide (DPN), 65% pure; 5.3 mgm. triphosphopyridine nucleotide (TPN), 80% pure; and, 3.6 mgm. coenzyme A (CoA), 75% pure. The stock solution is thawed just before it is added to the medium.

Preparation of One Liter of Synthetic Medium 703

The various constituents of the final medium (as also the ingredients of solution 1) are mixed in a 5-liter distillation flask with a large central and a smaller vertical side neck. The large central neck accommodates a bent glass stirring rod attached to a small motor, and the ingredients are added through the side neck. The stock solutions are combined as follows: Solution 1, 500 ml.; solution 2, 20 ml.; solution 8, 2 ml.; and 10 ml. each of solutions 3, 4, 5, 6, 7, and 9.

The final volume is adjusted to 1 liter by the addition of water, and the completed medium is sterilized by passage through UF fritted glass filters (Corning) and stored in the dark at room temperature. Just before use, 1 μgm. per ml. of sodium penicillin G is added.

Results

Solution 612

A year ago, it was observed that the synthetic media devised thus far were characterized by an unusually high oxidation-reduction potential. For this reason, a study was made of the effect on L strain mouse cells of greatly increased amounts of three reducing agents already present in solution 199, namely, cysteine, glutathione, and ascorbic acid. When the three substances were tested separately by addition to replicate cultures of washed suspensions of L strain cells in solution 199, the two containing sulphydryl (SH) groups, i.e., cysteine and glutathione, increased the rate of cell multiplication over that obtained in solution 199, improved the appearance of the cells, and caused them to live for longer periods. Higher levels of ascorbic acid had no apparent effect. When it was found that cysteine and glutathione could be used interchangeably, at the higher levels, cysteine was chosen because it could be obtained at lower cost. Then, ascorbic acid was added at a greatly increased level in the hope that it would help maintain the SH compounds in the reduced state. The levels of these substances that were finally adopted were as follows: cysteine hydrochloride, 26 mgm. %, and ascorbic acid, 5 mgm. %, which were, respectively, 2600 and 1000 times the levels present in solution 199. Eventually, glutathione, which is now known to be a coenzyme (9), was incorporated at 1 mgm. %, which is 200 times the level present in solution 199. The effect of solution 612 on washed suspensions of L strain cells is shown in Table II and in Fig. 1.

Solution 635

The pioneer work of Baker and Carrel (1), and of Fischer (5), suggested that the pronounced growth promoting activity of embryo tissue extracts resides mainly in the nucleoprotein fraction of the extracts. In order to determine

TABLE II

ESTIMATED NUMBER OF NUCLEI (IN THOUSANDS) IN REPRESENTATIVE SAMPLES OF ORIGINAL INOCULA AND IN REPLICATE CULTURES PREPARED FROM WASHED-CELL SUSPENSIONS OF L STRAIN MOUSE CELLS, AFTER 7 AND 12 DAYS IN VARIOUS SYNTHETIC SOLUTIONS

Replicate culture series	Source of nuclei counted	Nuclear counts for inoculum and 7-day cultures				Average inoc. and 7-day counts	Nuclear counts for 12-day cultures				Average 12-day counts	Identification of solutions
18492	Inoculum	427	482	369	479	439						
	Cultures in soln. 199	627	739	581	520	617	483	442	482	453	465	Soln. 199 (see text)
18461	Inoculum	327	441	390	426	396						
	Cultures in soln. 612	862	524	751		712	804	753	532		696	Soln. 612 is soln. 199 with more cysteine, glutathione and vit. C
	" " " 635	1073	813	1169		1018	1100	1024	955		1026	Soln. 635 is soln. 612 without the nucleic acid derivatives
18562	Inoculum	113	152	143	124	133						
	Cultures in soln. 652	697	602	758	653	673	730	876	905	680	796	Soln. 652 is soln. 635 with highly polymerized DNA (1 mgm.%)
	" " " 686	654	630	515	579	594	653	739	651	486	632	Soln. 686 is soln. 635 with an enzymic hydrolyzate of DNA (1 mgm.%)
18582	Inoculum	294	334	311	335	318						
	Cultures in soln. 635	1725	1451	1224	1265	1416						
	" " " 693	1919	1532	1587	1697	1683						Soln. 693 is soln. 635 with coenzyme concentrate (2 mgm.%) and vit. B_{12}
18594	Inoculum	94	136	91		107						
	Cultures in soln. 697	654	809	874	772	775	842	949	830	752	843	Soln. 697 is soln. 635 with coenzyme concentrate (1 mgm.%)
	" " " 698	553	661	824	572	653	959	861	839	884	886	Soln. 698 is soln. 635 with coenzyme concentrate (10 mgm.%)
18718*	Inoculum	258	253	268		260						
	Cultures in soln. 635	956	900	920	955	933	1263	1023	1107	1170	1141	
	" " " 703	1160	1250	1367	1154	1233	1090	1019	1254	1251	1154	Soln. 703 is soln. 635 with purified CoA, DPN, and TPN
18629	Inoculum	192	206	224	177	199						
	Cultures in soln. 635	1058	876	730	862	882	735	891	719	859	801	
	" " " 713	1213	1279	1043	1015	1138	859	1043	933	795	908	Soln. 713 is soln. 703 with 5 mgm.% creatine, 10 mgm.% phosphocreatine, 10 mgm.% ATP, and 20 μgm.% vit. B_{12}

** This series included 20 cultures; the other series shown included 48.*

the effect of certain nucleic acid constituents, a new basal medium, solution 635, was devised by omitting from solution 612 all the purines (adenine, guanine, xanthine, and hypoxanthine) and pyrimidines (thymine and uracil), as well as ATP, adenylic acid, ribose, and desoxyribose. This medium gave a much better growth response than solution 612, but although a three- to fourfold increase in the cell population of replicate L strain cultures was observed in one week (Table II), the cultures rarely survived longer than 40 days (Fig. 2). In the meantime, however, the medium did serve to excellent advantage as a basal medium for use in eliminating those nucleic acid constituents of solution 612 that were undoubtedly toxic. Thus, when solution 635 was supplemented with individual purines and pyrimidines at the conservative level of 10 μgm. per ml., and tested in replicate cultures assayed by DNAP determinations (7), adenine and guanine were markedly toxic, as were also thymine and uracil; but cytosine was nontoxic. Also, replicate culture DNAP assays made with the individual ribose nucleosides showed adenosine, alone, to be toxic, whereas all the ribose nucleotides were nontoxic. Neither intact ribonucleic acid (RNA) nor its enzymic hydrolyzate showed any effect on cell multiplication. In contrast, solution 652 (Fig. 3) containing highly polymerized calf thymus desoxyribonucleic acid (DNA), or oligonucleotides resulting from an enzymic hydrolyzate of thymus DNA incorporated in solution 635 at the same level (solution 686) gave four- and fivefold increases in the cell populations of replicate cultures in one week (Table II). The same response was also obtained with homologous DNA prepared from large populations of the L strain cells by the method of Marko and Butler (10). The effects of the desoxyribose nucleosides and nucleotides are now being investigated.

Solution 703

In a study of the effect of certain coenzymes, basal solution 635 was supplemented with Armour's porcine liver coenzyme concentrate over a wide range of concentrations. Cultures of L strain cells in solution 635 to which the concentrate was added at levels of 1 mgm. % (for solution 697) and 2 mgm. % (for solution 693) yielded five- and sixfold increases in their cell

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FIGS. 1 to 6. Living L strain (Earle) mouse cells in D-3.5 Carrel flasks after various periods of cultivation in different nutrient media. Magnification 120 ×.

FIG. 1. Culture 18375-1, after 20 days in solution 612. Almost all cells are abnormal, and many are dead.

FIG. 2. Culture 18632-3, after 20 days in solution 635. All cells are abnormal; the population is sparse.

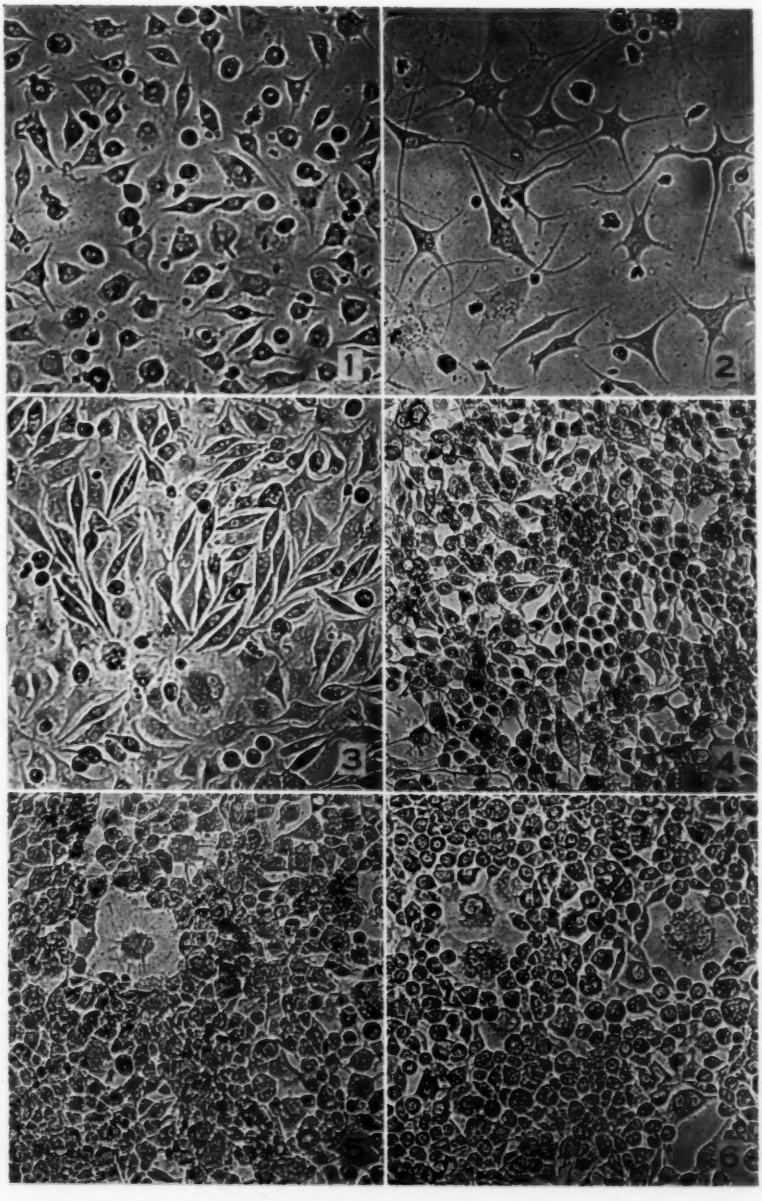
FIG. 3. Culture 18486-8, after 20 days in solution 652. Almost all cells are healthy; many mitoses.

FIG. 4. Culture 18585-8, after 183 days in solution 697. Population exceedingly healthy, with normal distribution of giant cells and many mitoses.

FIG. 5. Culture 18612-4, after 156 days in solution 703. Population exceedingly healthy, with giant cells and many mitoses.

FIG. 6. Culture 18698-3, after 61 days in 40% horse serum, in a balanced salt solution containing glucose (compare with Fig. 5).

PLATE I



populations in seven days (Table II) and have continued to remain in good condition for six months (Fig. 4). The active coenzymes known to be present in the crude concentrate are coenzyme A (CoA), diphosphopyridine nucleotide (DPN), and triphosphopyridine nucleotide (TPN). When these three coenzymes in a purified state, were added individually to solution 635, they had no effect. But when they were tested together at levels of 36 μgm., 78 μgm., and 42 μgm. per 100 ml., respectively (for solution 703), they caused approximately the same rate of cell multiplication in short-term replicate culture assays as was obtained with the crude concentrate (Table II). Even more important is the fact that two long-term test cultures prepared from washed suspensions of L strain cells have remained alive and healthy and still contain many dividing cells after five months in solution 703, having had complete fluid renewals twice a week (Fig. 5). In appearance, these cultures resemble those prepared from the same strain treated with horse serum and balanced salt solution containing glucose (Fig. 6). It would seem, therefore, that the effectiveness of the crude coenzyme concentrate resulted from the combined action of the three coenzymes tested, and not from extraneous material that may have been present in the concentrate. An experiment has also been made with acetyl CoA, prepared by the acylation of CoA with acetic anhydride at 0° C. The progress of acylation was followed by the disappearance of the SH groups of the CoA, as measured by the method of Grunert and Phillips (6). Replicate L strain cultures were not improved by the use of acetyl CoA in the presence of DPN and TPN.

Discussion

In the present communication, descriptions are given of further elaborations that have been made in a chemically-defined medium for the multiplication and survival of animal cells in tissue culture. The most of the improvements reported have been directed towards the stimulation of intracellular enzymes.

A distinct improvement in the ability of L strain mouse cells to multiply in synthetic media was observed upon the addition of greatly increased levels of cysteine and glutathione (solution 612). Singer and Barron (19) have demonstrated a protective action of added SH compounds for enzymes containing fixed SH groups that are essential for their activity. It is believed that the trace metals contributed by various ingredients of solution 199 (8) may combine with intracellular enzymes to form inhibiting mercaptides, and that the addition of higher levels of SH compounds may reverse this inhibition.

The elimination of the nucleic acid derivatives from solution 612, to give solution 635, had a further beneficial effect on cell multiplication. This observation confirmed the findings of Brachet (2), who demonstrated the toxic effects of yeast and muscle adenylic acids, guanylic acid, and ATP in explants of *Amblystoma* that were cultivated in saline solutions supplemented with

these compounds. Also, Williams (20) has shown that adenine, adenosine, and ATP are all competitive inhibitors of malic dehydrogenase *in vitro*, and discusses the possibility that adenine may inhibit still other enzymes.

The present experiments have also demonstrated that the addition of purified CoA, DPN, and TPN to basal solution 635 (to make solution 703) resulted in a greatly increased rate of cell multiplication of L strain mouse cells and yielded cell populations of these cells that have survived for almost five months. The beneficial effect of CoA is in keeping with many biochemical reactions that are now known to be mediated by this coenzyme (14), which occupies a key position in the metabolism of carbohydrates, fatty acids, and amino acids. Acetyl CoA, for example, is required to initiate the Krebs cycle, which is a far more efficient source of energy for biosynthesis than is glycolysis. Also, the generation of acyl CoA by oxidative decarboxylation of α-keto acids requires the participation of an electron acceptor (15), which could account for the synergistic effect of the three coenzymes tested in the present experiments. In any event, L strain cultures treated with synthetic media that did not contain these coenzymes showed active cell multiplication during the first 7–12 days of cultivation, but thereafter underwent a progressive process of degeneration until, after about 40 days, the cultures were usually dead. It may well be that tissues cultivated in synthetic media are gradually depleted of certain coenzymes by a process of slow dialysis, when these coenzymes are not present in the media. Even when the coenzymes are present, it becomes necessary to determine the optimal frequency with which the culture media should be renewed, if the utmost advantage is to be taken of their beneficial effects on cell multiplication and survival. This problem is now being investigated.

Acknowledgments

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STUDIES ON THE PRESERVATION OF BLOOD

III. REVERSIBLE INHIBITION OF GLYCOLYSIS IN BLOOD BY OXALATE[1]

By Hanna M. Pappius[2] and Orville F. Denstedt

Abstract

Oxalate was found to inhibit glycolysis in red blood cells during storage at 5° C. The inhibition of glycolysis in blood cells by oxalate is reversible. The glycolytic activity of the oxalated cells can be restored by washing the cells, or by precipitating the oxalate with an equivalent concentration of added calcium ions. However, with increased duration of storage prior to removal of oxalate the rate of glycolysis in the erythrocytes was found to be decreased on removal of the oxalate.

Introduction

Oxalate, in addition to preventing the clotting of blood plasma, is known to inhibit the enzyme enolase (2) and to inhibit or retard the glycolytic activity of erythrocytes (3). The present communication concerns the reversibility of the inhibition by oxalate, and the influence of storage of the red cells in the inactivated state for various periods of time, on the capacity of the cells to recover glycolytic activity upon removal of the oxalate.

Methods

The method of collection and storage of the blood specimens and the analytical procedure were the same as described in an earlier communication (4).

In the experiments to be described isotonic potassium oxalate (1.85%) solution was used as the diluent. The proportion of blood to oxalate by volume was 7 : 1 in some cases, 2 : 1 in others. Since the glycolytic behavior was found to be influenced by the degree of dilution of the blood sample the results obtained with two dilutions will be reported.

One experiment was designed to study the reactivation of the glycolytic system when oxalate-treated cells are suspended in oxalate-free serum. Five aliquots of blood were collected into isotonic oxalate solution in the proportion of 2 parts of blood to 1 part of isotonic potassium oxalate solution by volume. At the same time serum was obtained from a defibrinated specimen of blood from the same donor. The samples were stored at 5° for five days after which time one aliquot was reserved as a 'control' specimen while the cells in the other four aliquots were washed twice with isotonic saline and resuspended in

[1] *Manuscript received February 2, 1954.*

Contribution from Department of Biochemistry McGill University, Montreal, Que. The material in this paper is taken from theses submitted by H. M. Pappius in partial fulfillment of the requirements for the degree of Master of Science (1948) and of Doctor of Philosophy (1952) in Biochemistry at McGill University.

[2] *Present address: Montreal Neurological Institute, Montreal, Que.*

the following media, care being taken to ensure uniform dilution of the cells with the serum:

A. Oxalated plasma (O). In this 'control' sample the cells were left in the oxalated plasma.
B. Serum (S).
C. Serum to which calcium lactate was added to give final concentration of calcium of approximately 12 mgm. per 100 ml. (SCa).
D. Serum to which magnesium lactate was added to give final concentration of magnesium of approximately 6.5 mgm. per 100 ml. (SMg).
E. Serum to which both magnesium lactate and calcium lactate were added in amounts as above (SCaMg).

All the samples were placed again in the refrigerator and the change in the glycolytic activity was ascertained by periodic analyses in the usual manner.

Another experiment was carried out to study the effect of removing the oxalate by the addition of calcium at various times during storage. Three aliquots of blood were collected, defibrinated, and treated as follows:

F. To one defibrinated sample isotonic potassium oxalate solution was added in the proportion of 1 volume of oxalate to 4 of blood. After storage for five days at 5° C. an amount of calcium acetate, equivalent to the oxalate present in the sample, was added in a small volume of distilled water. (To 40 ml. defibrinated blood 10 ml. of the oxalate solution was added, followed by the addition of 175 mgm. calcium acetate dissolved in 2 ml. of water. This gave final dilution of blood, by volume, of approximately 5 to 1.5).
G. The second defibrinated sample was treated exactly as described above, except that calcium acetate was added on the ninth day of storage.
H. To the third defibrinated specimen potassium acetate was added in amount comparable to that present in samples F and G, and so as to give the final proportion, by volume, of 5 parts of blood to 1.5 parts of potassium acetate solution. (To 40 ml. defibrinated blood 12 ml. of solution containing 218 mgm. potassium acetate were added.) This was done to test the effect, if any, of the potassium acetate formed when calcium acetate was added to specimens F and G to precipitate the oxalate.

The chemical changes were followed in the three samples in the usual manner during storage at 5° C.

Results

The Effect of Oxalate on the Glycolytic Changes in Blood During Storage at 5° C.

The results of two experiments with oxalated blood, typical of a total of four, are tabulated in Table I.

It is evident from Table I that in the specimen in which the ratio of blood to 1.85% potassium oxalate solution, by volume, was 7 : 1 the breakdown of glucose and the accumulation of lactic acid were greatly diminished

TABLE I

THE BEHAVIOR OF GLUCOSE, LACTIC ACID, PYRUVIC ACID, AND DIPHOSPHOGLYCERATE IN OXALATED BLOOD DURING STORAGE AT 5° C.

Duration of storage, days	Millimoles per liter			
	Glucose	Lactic acid	Pyruvic acid	Diphosphoglycerate
Proportion of blood to 1.85% potassium oxalate solution, by volume, of 7 : 1				
0	4.52	1.76	0.096	2.54
3	3.22	2.25	0.091	2.54
6	3.38	3.26	0.062	2.64
9	2.88	3.67	0.056	2.67
12	2.88	3.85	0.068	2.28
15	2.73	3.56	0.110	2.89
18	2.61	5.40	0.278	2.61
21	2.73	4.80	0.522	2.61
24	2.50	5.25	0.734	2.75
Proportion of blood to 1.85% potassium oxalate solution, by volume, of 2 : 1				
0	4.11	0.88	0.051	1.55
2	3.55	1.11	0.039	1.63
5	3.22	1.22	0.051	1.45
8	3.00	1.55	0.028	1.50
11	3.05	1.88	0.073	1.34
14	2.88	2.27	0.085	1.46
18	3.05	2.77	0.170	1.64
22	3.11	2.11	0.210	1.58

compared with that in citrated blood (5). The concentration of diphosphoglycerate was gradually increased and the accumulation of pyruvate, which did not commence until the third week of storage, was gradual.

It is also evident from the data in Table I that an increase in the amount of oxalate added to the blood (proportion 2 volumes oxalate to 1 of blood) produced a more complete inhibition of glycolysis. Thus approximately only one millimole of glucose was broken down (compared with two millimoles in the former case) and the accumulation of lactate was correspondingly diminished. No significant change in the level of diphosphoglycerate was observed throughout the period of 22 days, while the accumulation of pyruvate, by the third week of storage, was only a third of that observed in the former case.

The Effect of Suspending Oxalate-treated Cells in Oxalate-free Serum

When oxalated cells were washed with saline to remove the oxalate and then resuspended in media free from oxalate, the capacity to metabolize

TABLE II

THE EFFECT OF SUSPENDING OXALATE-TREATED CELLS IN OXALATE-FREE SERUM, WITH AND WITHOUT THE ADDITION OF MAGNESIUM AND CALCIUM IONS, ON THE BEHAVIOR OF GLUCOSE, LACTIC ACID, PYRUVIC ACID, DIPHOSPHOGLYCERATE, AND INORGANIC PHOSPHATE IN BLOOD DURING STORAGE AT 5° C.

Duration of storage, days	Millimoles per liter of whole blood																									
	Glucose					Lactic acid					Pyruvic acid					Diphosphoglycerate					Inorganic phosphate				
	Sample*																									
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
	Suspension medium																									
	O	S	SCa	SMg	SCaMg	O	S	SCa	SMg	SCaMg	O	S	SCa	SMg	SCaMg	O	S	SCa	SMg	SCaMg	O	S	SCa	SMg	SCaMg
0	3.28	3.22	3.56	3.50	3.11	1.83	1.33	3.16	2.94	3.72	0.034	0.051	0.045	0.051	0.051	1.05	0.88	0.84	0.42	0.74	1.95	0.84	0.84	0.84	0.90
3	2.97	2.61	2.33	2.33	1.97	1.38	2.50	4.00	3.88	4.83	0.017	0.057	0.057	0.040	0.028	1.29	0.87	0.97	0.82	1.29	1.84	0.74	0.79	0.68	0.63
7	2.94	2.11	1.72	1.78	1.33	1.88	4.44	6.11	5.66	6.88	0.011	0.057	0.051	0.051	0.034	1.82	1.00	0.89	0.84	1.26	2.16	1.00	1.00	1.00	1.00
12	2.94	1.58	1.06	1.11	0.44	2.27	5.72	7.05	6.77	8.16	0.034	0.091	0.080	0.068	0.051	1.37	0.87	0.84	0.94	1.16	2.47	1.32	1.63	1.37	0.84
16	3.00	1.11	0.78	0.89	0.17	2.33	7.33	9.11	8.55	8.55	0.011	0.023	0.040	0.034	0.074	1.08	—	—	—	1.00	2.53	—	1.63	—	1.84
20	2.72	—	—	—	0.39	2.44	—	—	—	9.11	0.091	—	—	—	0.341										

** All blood samples were collected in isotonic potassium oxalate solution (1.85%) (volume of blood to oxalate 2 : 1), and stored at 5° for five days. On the fifth day cells of sample A were left in oxalate (O), while the cells of the other four samples were washed and resuspended as follows: sample B—in serum (S); sample C—in serum fortified with Ca (SCa); sample D—in serum fortified with Mg (SMg); sample E—in serum fortified with Ca and Mg (SCaMg). For details see text. Duration of storage in the table is given from the time at which the above additions were made, namely on the fifth day of storage.*

TABLE III

THE EFFECT OF REMOVAL OF OXALATE, BY ADDITION OF CALCIUM ACETATE, AT VARIOUS STAGES OF STORAGE ON THE BEHAVIOR OF GLUCOSE, LACTIC ACID, PYRUVIC ACID, DIPHOSPHOGLYCERIC ACID, INORGANIC PHOSPHATE, AND HYDROLYZABLE PHOSPHATE IN WHOLE BLOOD AT 5° C.

Duration of storage, days	Millimoles per liter of whole blood																	
	Glucose			Lactic acid			Pyruvic acid			Diphosphoglyceric acid			Inorganic phosphate			Hydrolyzable phosphate		
	Sample*																	
	F	G	H	F	G	H	F	G	H	F	G	H	F	G	H	F	G	H
	Day of addition of calcium acetate																	
	5	9	—	5	9	—	5	9	—	5	9	—	5	9	—	5	9	—
0	3.55	3.55	2.44	2.83	3.00	2.44	0.057	0.045	0.045	1.92	1.70	1.44	1.10	1.10	0.84	1.16	1.16	1.42
3	3.55	—	1.28	3.33	—	5.27	0.040	—	0.051	1.89	—	1.63	1.37	—	0.68	0.94	—	1.11
5	2.83	3.55	0.33	3.55	3.16	6.88	0.045	0.040	0.045	1.86	1.73	1.55	1.58	1.37	0.89	0.79	1.00	1.53
—	2.53	—	—	3.66	—	—	0.034	—	—	1.63	—	—	1.63	—	—	0.84	—	—
8	1.33	—	0.66	5.72	—	7.00	0.045	—	0.028	1.58	—	1.34	1.37	—	1.84	1.21	—	0.63
9	—	3.05	—	—	2.94	—	—	0.040	—	—	1.68	—	—	1.74	—	—	0.68	—
—	—	2.89	—	—	2.66	—	—	0.040	—	—	1.63	—	—	1.47	—	—	0.58	—
12	0.44	1.78	0.39	7.44	3.50	6.61	0.136	0.119	0.511	1.50	1.58	0.66	2.58	2.00	3.79	0.31	0.42	0.31
16	0.28	0.94	—	7.11	5.22	—	0.794	0.261	—	0.66	1.23	—	4.10	2.42	—	0.00	0.32	—
21	0.17	0.78	—	7.94	6.33	—	0.976	0.256	—	0.58	1.21	—	4.90	2.89	—	0.00	0.00	—

* *All blood samples were defibrinated. Isotonic potassium oxalate (1.85%) was added to samples F and G after defibrination (proportion of 1 volume of oxalate to 4 of blood). Calcium acetate was added on fifth day to sample F, to precipitate the oxalate, and on the ninth day to sample G. Sample H contained potassium acetate from the beginning of storage. For experimental details see text.*

glucose was restored, while in the oxalated control no utilization of glucose occurred. The suspension medium was serum or serum containing added Mg^{++} and/or Ca^{++}, since these ions are either removed or their concentration is decreased by oxalate.

The rate of glucose utilization differed in the four experimental samples B to E, as is evident from the data in Table II. The fall in the concentration of glucose was smallest in the cells resuspended in serum alone (B), greater in cells resuspended in serum to which either Mg^{++} or Ca^{++} had been added (C and D), and greatest in the sample containing the added Ca^{++} and Mg^{++}. However, the magnitude of the differences is of doubtful significance. The difference in behavior may be the result of the differences in the degree of dilution of the cells in these samples. Despite the care taken to ensure a uniform concentration of the cells by regulating the quantity of diluent added, the hemoglobin concentration in the four samples was, respectively, as follows, in gm. %: (B), 6.33; (C), 7.37; (D), 7.17; (E), 9.87.

Lactic acid accumulated throughout the storage period in the four oxalate-free samples (B-E), while the increase in the concentration of lactate in the control (oxalated) sample was comparatively very small (cf. Table II). The relatively higher initial concentration of lactic acid in samples C, D, and E was due to the addition of magnesium and calcium lactate at the beginning of the experiment. An increase in the lactate concentration within this range has no effect on the rate of glycolysis in blood (4).

No significant change in the concentration of pyruvic acid and diphosphoglyceric acid (cf. Table II) occurred in any of the samples throughout the experimental period of 16 days. A rise in the concentration of pyruvate was observed, however, in sample E on the 20th day, at which time all of the glucose had been used up in that sample.

In all the samples the concentration of inorganic phosphate fell slightly on the third day and thereafter showed a slow but steady upward trend until the end of the experiment (cf. Table II). Since no breakdown of diphosphoglycerate occurred during that time, the accumulation of inorganic phosphate signifies the gradual breakdown of the more "labile" phosphate esters, mainly ATP.

The Effect of Removal of Oxalate by Addition of Calcium at Various Times During Storage of Oxalated Blood Specimens

It was of interest to determine whether the method of removal of oxalate has any effect on the degree of restoration of the glycolytic activity of blood preserved in oxalated medium, and to ascertain whether the time when the oxalate is removed affects the glycolytic capacity of the cells. Accordingly on the fifth and ninth day of storage the oxalate was removed from defibrinated and oxalated specimens by the addition of calcium.

It is evident from Table III that in the oxalate-free control sample H, glucose was metabolized and reached the minimum concentration by the fifth day of storage. In the two samples F and G containing oxalate the utilization

of glucose was inhibited. In sample F, after the oxalate was precipitated on the fifth day, the glucose was rapidly utilized until the 12th day by which time the sugar had been almost depleted. In sample G, on removal of oxalate on the ninth day, the glucose utilization proceeded at about the same rate as in the other samples until the 12th day, after which time the rate gradually decreased. By the end of the 21-day experimental period the glucose content in sample G had not yet been exhausted. It is apparent from the data in Table III that the changes in the concentration of lactic acid corresponded closely with those of the glucose.

In Table III are summarized also the observations with reference to pyruvate and diphosphoglycerate. In all samples the concentration of both the pyruvate and diphosphoglycerate remained unchanged during the first week of the experiment. In sample H the diphosphoglycerate concentration fell abruptly and pyruvate accumulated after the eighth day when the glucose had been exhausted. Similar changes were observed in sample F after the 12th day, when glucose reached its minimum value in that sample. In sample G also the diphosphoglycerate fell and pyruvate accumulated after the 12th day, despite the presence of glucose, but the rates of these changes were much smaller than in samples F and H.

It is evident from the data in Table III that the accumulation of inorganic phosphate in the three samples F, G, and H reflected the breakdown of diphosphoglycerate. It is apparent also that the concentration of the hydrolyzable phosphate, or the phosphate derived from two groups of ATP and one group of hexosediphosphate, decreased steadily in the three samples throughout the experimental period. The difference between the behavior of the control and the experimental specimens was not significant.

Discussion

The results obtained in our experiments with oxalated blood during storage at 5° C. are consistent with the observation of other workers that the dephosphorylation of phosphoglycerate is inhibited by oxalate (2). Furthermore, the inhibition is reversible. It is not possible to establish from the results of the studies whether the restoration of glycolytic activity is attributable to the added magnesium or to the calcium ions. Restoration of glycolytic activity was produced by resuspending the cells in normal serum which contains both calcium and magnesium ions. These latter ions are of special interest as they are essential to the activity of enolase. In this connection Fishman (1) has made the interesting observation that the magnesium concentration in blood is not altered by the addition of oxalate. It is possible, however, that the concentration of magnesium ions may be diminished by formation of a complex with the oxalate and thus may inhibit or interfere with the activity of enolase. There is an alternative possibility also that oxalate may interfere directly in some unknown way.

The length of the period during which the cells are kept in the presence of oxalate appears to influence the degree to which glycolysis can be restored in

oxalated blood. If oxalate was added to blood and removed within the first week of storage, the restored glycolytic activity was found to be comparable to that in a citrated control specimen (5). If removal of the oxalate was delayed until the ninth day the changes observed were somewhat similar to those in blood stored in citrate–glucose medium (4). It appears that oxalated blood, like citrated specimens, suffers an impairment of the glycolytic mechanism about the end of the second week of storage and in both cases the nature of the alteration resembles that which occurs in the red cells preserved in the glucose containing mixture.

The changes that occur in the concentration of the organic phosphate compounds suggests that much of the breakdown of ATP in red blood cells during storage occurs independently of glycolysis, since little difference was observed in the behavior of the phosphate esters between samples in which glycolysis was inhibited by the addition of oxalate and those which were actively glycolyzing. However, the ATP level is maintained for a longer period in preserved red cells when an excess of glucose is present, i.e. citrate–glucose preservative media (4).

It should be noted that the rate and extent of breakdown of glucose were considerably smaller in the samples containing washed cells than in those from which oxalate was removed by precipitation. While no definite conclusion has been drawn on the strength of these results alone, it is possible that the diminished glycolytic activity of the washed cells may be in some way connected with the washing treatment, as in our experience washing of the cells has a detrimental effect on the stability of the cells.

Acknowledgment

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